CURRICULUM VITAE
ANN SODJA, Ph.D.

Address: Department of Biological Sciences
3105 Biological Sciences Bldg.
Wayne State University
5047 Gullen Mall
Detroit, MI 48202-3917

Telephone Nos.: (313) 577-2908 (Office)
(313) 577-2925 (Laboratory)

Birthplace: Ljubljana, Slovenia
Citizen of U.S.A.

Education:

Baccalaureate: A.B. in Chemistry with honors, Ursuline College, Cleveland, Ohio, 1962.

Graduate: M.S. in Biochemistry, the Ohio State University, Columbus, Ohio, 1964.

Doctoral: Ph.D. in Biochemistry, University of California, Davis, California, 1974. (Advisor: Dr. Paul K. Stumpf).

Postdoctoral California Institute of Technology, Pasadena, California, 1974-78. (Postdoctoral Mentor: Dr. Norman Davidson)

Professional Appointments: Tenured Associate Professor, Wayne State University, 1983.
Associate Professor, Wayne State University, 1982.
Assistant Professor, WSU, 1978.

Professional Society Memberships:
AAAS, 1978-present.
Sigma Xi, 1978-present.
Association for Women in Science (AWIS), Detroit Chapter 1986 - present.
AWIS, National Organization, 1988 - present.

Honors/Awards:
National Honor Society and Dean's List (all throughout undergraduate education, 1958-62).
A.B. cum laude, 1962.
Charles Kettering Predoctoral Fellowship, 1962-1963.
NIH Predoctoral Traineeship, 1963-1965.
Max-Planck Fellowship for Visiting Scientists, 1967-1968.

NSF Predoctoral Fellowship, 1969-1974.
American Cancer Society Postdoctoral Fellowship, California Division, 1974-1976.
Research Fellow in Chemistry, California Institute of Technology, 1976-1978.
Career Development Chair Award, WSU, 1984.
Presidential Excellence Award, WSU, 1986, 1987 & 1990.
Women of Wayne Staff/Faculty Recognition Award, honorable mention, 1989.
Nominated by the students for the President's Award for Excellence in Teaching 1990, 1991.

Teaching Experience

Years at Wayne State: 20.
Years at Other Colleges/Universities
Ohio State University, 1962-64, Graduate T.A.
University of California, Davis, 1970-71, Graduate T.A.

Research

Fellowships:

Graduate:

Ann Sodja, Charles Kettering Predoctoral Fellowship, 1962-63, tuition plus stipend.
Ann Sodja, NIH Predoctoral Traineeship, 1963-65, tuition plus stipend.
Ann Sodja, NSF Predoctoral Fellowship, 1969-74, tuition plus stipend.

Postdoctoral:

Ann Sodja, Isolation of Duck Hemoglobin Genes Using Biochemical Gene Enrichment Techniques, 1974-76, American Cancer Society Fellow, California Division, approximate stipend.
Ann Sodja, Cloning and characterization of tRNA and 5S RNA of Drosophila melanogaster, 1976-78, Research Fellow in Chemistry, California Institute of Technology, approximate stipend.

Grants:

Federal:

Ann Sodja, Co P.I., Contractile Protein Gene Expression During Development, 1981-85, NIH.
Ann Sodja, Assoc. P.I., Contractile Protein Gene Expression During Development, following award periods:
1982 (All these are subprojects in the University).
1983 (wide proposal to NIH, Division of Research).
1984 Resources, (Prof. Dunbar, PI).

1985.
1986, (Each year's budget runs from 1/1-12/31 of the same year).
1987, 1988, 1989 & 1990.

Grants (All grants listed below are/were funded unless indicated otherwise.)

Federal:

Ann Sodja, Assoc. P.I., Actin Genes: Evolution of Their Structure and Regulation, GM08167, 1/1/91-1/1/95.
1991 (This project was a subproject of the University wide proposal to the NIH, NIGMS (Prof. Dunbar, P.I.). Each years budget runs from 1/1-12/31 of that year.)
1992, 1993 & 1994 (2/28/95).

Ann Sodja, Acting P.I., DNA Fingerprinting and Paternity Assessment in Non-Human Primates, NSF BNS 88-18405, 7/31/90 - 7/31/92.
Ann Sodja, Co P.I., Research Careers for Minority Scholars, NSF, W. Rolnick, PI (Physics), 1990-1995.
NIH, NIGMS, MARC training program continuation renewal; Faculty Associate in the program. Prof. Jay, P.I. Funded for 6/1/92 - 5/3/97.
Ann Sodja, Co P.I., A. Rosenspire, P.I., Proposal for Research Experience for Undergraduates, NSF, 1987 & 1988 (A/NF).

Private:

Ann Sodja, Characterization of Genes Coding for Actin in Drosophila melanogaster, 1979-80, Muscular Dystrophy Association (MDA).
Ann Sodja, Characterization of Genes Coding for Actin in Drosophila melanogaster, 1980-81, MDA.
Ann Sodja, Chromatin Structure During Actin Gene Expression, 1982-1983, MDA.
Ann Sodja, Regulation of Actin Gene Expression, American Heart Association (MI.), 1/7/87-6/30/88.
Sept. 9, 1987, Regulation of Actin Gene Expression, American Heart Association (MI), 1/7/88-6/30/90, (A/NF).

Grants Pending revisions/review/funding:

June 1, 1989, Actin Genes: Evolution of Their Structure and Regulation, Ann Sodja, PI, 33%, N1H,1RO1GM43785-01, (TDC), 4/1/90 - 3/31/95, (A/NF).
December 1, 1989, Actin Genes: Evolution of Their Structure and Regulation, Ann Sodja, PI, 33%, NSF, DMB-9003727, (TDC), 7/1/90-6/30/93, (A/NF).
March 22, 1991, Actin Gene(s) in Drosophila virilis, Ann Sodja, PI, 10%,

Research Award Women of Wayne Alumni Association, 1991-92, (A/NF).

October 14, 1991, Supplemental Research Equipment Fund, WSU, submitted jointly with Prof. Arking, (NF).

Grants in preparation:

New project on molecular biology of the mosquito.
(No funding information available at this time).

Other Fellowships:

Grant-in-Aid Awards, WSU-ORSPS (the ultimate source of money is NIH, Biomedical Research Division.):

Ann Sodja, Characterization of Actin Genes in Drosophila melanogaster, 1979-80.

Ann Sodja, Comparison of Actin Coding and Its Adjacent-Sequences in Evolution,1980-81.

Ann Sodja, Chromatin Structure during Actin Gene Expression, 1981-82.

Ann Sodja, Molecular Comparison of Actin Genes in Drosophila melanogaster & Musca Domestica, 1984-85.

Ann Sodja, Coordinate Control of Actin Gene Expression, 1985-86.
1985, on a noncompetitive basis.

August, 12, 1988, Evolution of Actin Genes and Regulation of Their Expression, 1988-89 (A/NF).

Faculty Research Awards:

Ann Sodja, Regulation of Actin Gene Expression, 1987-88.

Special Awards:

Ann Sodja, Biochemical Characterization of Collagen/Tropocollagen, 1967-68, MaxPlanck Gesellschaft, (Visiting Scientist's Fellowship)

Ann Sodja, Developmental Actin Gene Expression, 1984-86, Career Development Chair Award (WSU).

Ann Sodja, Actin Gene Regulation in Drosophila, 1986-89, Molecular Biology Center (WSU).

Ann Sodja, Postdoctoral Fellow Position Presidential Excellence Award, 1986.

Ann Sodja, Received in matching funds a second postdoctoral fellowship position from the Center of Molecular Biology (CMB), (WSU), 1986.

Ann Sodja, Postdoctoral Fellowship position from CMB (WSU), 1987.

CMB Predoctoral Fellowship for Christopher Horak, 1986-88.

Ann Sodja, Travel Award from L.A. College and VPR's office, 1987.

Ann Sodja, President's Excellence Award, 1987.

Ann Sodja, Postdoctoral Fellowship position from CMB (WSU), 1988.

Ann Sodja, matching funds for publication costs.
CMB Predoctoral Fellowship for Xisojun Yan, 1988-1989, and 1989-1990.
CMB salary support for Baolin Wang, 9/15/88-3/31/89.
January 2, 1990, Molecular Characterization of Actin Gene(s) in Drosophila virilis, Ann Sodja, PI, 33%, WSU Presidential Excellence Award through Biological Sciences.
May 13, 1991, Actin Gene(s) in Drosophila virilis, Ann Sodja, PI, 30%, Biomedical Research Support, 1991-1992.
December 5, 1995-1996. Research Stimulation Fund Grant to initiate molecular investigation on mosquito from the Office of the Vice President for Research and in matching funds from the Department of Biological Sciences.
March, 1996, Arthropod-Borne and Infectious Diseases Lab (AIDL), Ft. Collins, CO. Award- to cover expenses for attendance of Biology Disease Vectors. External $1,680 (MacArthur Foundation).
April, 1996, Research Development Award.
January 1999, Olfactory Receptors Guide Mosquitoes to their Blood Meal, Research stimulation grant, WSU.

Publications

Journal Articles Published (all years)

Taniuchi, H., C.B. Anfinsen, A. Sodja, The Amino Acid Sequence of an Extracellular Nuclease of Staphylococcus aureus, II. The Amino Acid Sequences of Tryptic and Chymotryptic Peptides, J. Biol. Chem., 242:4735-4751 (1967).

Taniuchi, H., C.B. Anfinsen, A. Sodja The Amino Acid Sequence of an Extracellular Nuclease of Staphylococcus aureus, III. Complete Amino Acid Sequence, J. Biol. Chem., 242:4752-4767 (1967).

Taniuchi, H., C.B. Anfinsen, A. Sodja, Nuclease-T: An Active Derivative of Staphylococcal Nuclease Composed of Two Noncovalently Bonded Peptide Fragments, Proc. Nat. Acad. Sci., 58:1235-1242 (1967).

Harwood, J.L., A. Sodja, P.K. Stumpf, A.R. Spurr, On the Origin of Oil Droplets in Maturing Castor Bean Seeds, Ricinus Communis, Lipids 6:851-862 (1971).

Harwood, J.L., A. Sodja, P.K. Stumpf, Beta Hydroxylation of Fatty Acids by a Soluble Preparation from Maturing Avocado Mesocarp, Biochem. J. 130:1013-1022 (1972).

Sodja, A., P.K. Stumpf, Fat Metabolism in Higher Plants: Metabolism of Medium Chain Fatty Acids, Lipids 10:818-828 (1975).

Yen, P.H., Sodja, A., Cohen, M. Jr., Conrad, S.E., Wu, M., Davidson, N., Ilgen, C., Sequence Arrangement of tRNA Genes on a Fragment of Drosophila melanogaster DNA Cloned in E. coli. Cell 11: 763-777 (1977).

Hershey, N.D., Conrad, S.E., Sodja, A., Yen, P.H., Cohen, M. Jr., Davidson, N., Ilgen, C., Carbon, J., The Sequence Arrangement of Drosophila melanogaster 5S DNA Cloned in Recombinant Plasmids. Cell 11: 585-598 (1977).

Sodja, A., Davidson, N., Gene Mapping and Gene Enrichment by the Avidin-Biotin Interaction: Use of Cytochrome-c as a Polyamine Bridge. Nucleic Acids Research 5: 385-401 (1978).

Davidson, N., Fyrberg, E.A., Hershey, N.D., Kindle, K., Robinson, R.R., Sodja, A., Yen, P., Recombinant DNA Studies of DNA Sequence Organization Around Actin and tRNA Genes of Drosophila in "RNA-Polymerases, tRNA and Ribosomes: Their Genetics and Evolution," S. Osawa, H. Ozeki, H. Uchida and T. Yura, Eds., University of Tokyo, p. 279-295 (1980).

Fyrberg, E.A., Kindle, K.L., Davidson, N. and Sodja, A., The Actin Genes of Drosophila: A Dispersed Multigene Family. Cell 19: 365-378 (1980).

Sodja, A., Arking, R., Zafar, R.S., Actin Gene Expression During Embryogenesis of Drosophila melanogaster. Develop. Biol. 90: 363-368 (1982).

Sodja, A., Rizki, R.M., Rizki, T.M., Zafar, R.S., Overlapping Deficiencies Refine the Map Position of the Sex-Linked Actin Gene of Drosophila melanogaster. Chromosoma 86: 293-298 (1982).

Zafar, R.S., Sodja, A., Homology in the Actin Coding and Adjacent Sequences in two Widely Divergent Species. Biochem. Biophys. Res. Comm. 111:67-73 (1983).

J. Papa Rao, Zafar, R.S., Sodja, A., Transcriptional Activity at the 3' End of the Actin Gene at 5C on the X Chromosome in Drosophila melanogaster. Biochim. Biophys. Acta, 950:30-44 (1988).

Wang, B., and Sodja, A., Alternate Approach to Sequencing Double-Stranded Template DNAs. BioTechniques 10: 198-201 (1991).

Winrow, M.A., and Sodja, A. Cloning, Initial Characterization of Several Actin Genes in the Parasitic Nematode, Ascaris suum. Biochem. Biophys. Res. Comm. 178: 578-585 (1991).

Rao, J.P., and Sodja, A. Further Analysis of a Transcript Nested Within Actin 5C Gene of Drosophila melanogaster. Biochem. Biophys. Res. Comm. 184:400-407 (1992).

Hadden, T.J., and Sodja A., An Oligogene Family Encodes Actins in the Housefly, Musca domestica, Biochem. Biophys. Res. Comm. 203:523-531 (1994).

Journal Articles (Submitted/In Press):

Hadden, T.J., and Sodja, A., A Housefly Actin Gene: Interspecies Conservation of Coding and Putative Regulatory Sequences.

Journal Articles in Preparation:

Wang, B.L., Liu, G., and Sodja, A. An Actin Gene of Drosophila virilis.

Sodja, A., Fujioka, H., Lemos, F.J.A., Donnely-Doman, M., and Jacobs-Lorena, M. (2000). The Induction of Actin Gene Expression in the Mosquito Midgut in Response to Blood Ingestion Correlates with Dramatic Changes of Cell Shape. Manuscript to be submitted shortly.

Abstracts Published:

Sodja, A., Hershey, N.D., Conrad, S.E., Davidson, N., Ilgen, C., Carbon, J., Mapping Drosophila 5S Genes in Several Recombinant Plasmids. J. SUPRAM ST1977 (S1):53 Abstract from the ICN-UCLA Symposia on the Molecular Approaches to Eucaryotic Genetic Systems (1977).

Sodja, A., Isolation and Characterization of Actin Genes in Drosophila melanogaster. In Gene Structure and Expression (Ohio State University Biosciences Colloquia, No. 6), D.H. Dean, L.F. Johnson, P.C. Kimball, R.S. Perlman, Eds., The Ohio State University Press (1980).

Sodja, A., Zafar, R.S., Arking, R., Differential Actin Gene Expression in Drosophila melanogaster. Abstract of the Cleveland Symposium on Macromolecules, International Congress on Recombinant DNA, Elsevier Publishing Co., p. 32 (1981).

Sodja, A., Zafar, R.S., Mildner, A.M., Actin Gene Expression During Development of Drosophila melanogaster. Abstract of the Symposium Presented at the 149th National Meeting of the American Association for the Advancement of Science, Detroit, Michigan, AAAS Abstracts, p. 42 (1983).

Sodja, A., Zafar, R.S., Developmentally Linked transcription at the Sex-Linked Actin Gene in Drosophila melanogaster. Abstract of the Poster Session Presented at the UCLA Symposium on Molecular Biology of Development held in April, 1984 at Steamboat, Colorado. Abstract in J. Cell. Biochem., Supplement 8B, p. 50 (1984).

Sodja, A., Mildner, A.M., Elsenboss, L., Actin Genes in Different Drosophila Species, abstract of the poster session presented at UCLA symposium on Molecular Biology of Muscle Development held in March, 1985 in Park City, Utah. Abstract in J. Cell. Biochem. Supplement 9B, p. 53, 1985.

Winrow, M.A., Martin, P.E., Sodja, A., Preliminary Study of Actin Genes in Ascaris suum, abstract of a poster presented at the 13th Annual MBRS Symposium held in Miami, Florida, April, 1985. Abstracts #104, p. 18.

Elsenboss-Pena, L., Sodja, A., Only 3 Genes Encode Actins in Drosophila virilis, abstract of the poster presented at UCLA Symposium on Molecular Entomology, J. Cell. Biochem. Supplement lOC, p. 72 (1986).

Winrow, M.A., Martin, P.E., Sodja, A. A Study of Actin Genes in Ascaris suum, abstract of the poster presented at UCLA Symposium on Molecular Biology of Invertebrate Development, J. Cell. Biochem. Supplement llC, p.31, 1987.

Zafar, R.S., J. Papa Rao, Sodja, A. A Unique Configuration at the 3' End of Actin Gene of Drosophila melanogaster, 18th FEBS meeting Abstract TU 2.2.5, p. 24, 1987.

Sodja, A., J. Papa Rao, Zafar, R.S. An Actin Associated 3' UTR in Drosophila Contains a Transcriptional Unit. UCLA Symposium on Molecular Biology of RNA, J. Cell. Biochem., 12C, N720, 1988.

Hadden, T.J., Sodja, A. The Actin Genes of Musca Domestica. 4th International Congress of Cell Biology, Abstracts, #8.4.26, p. 303, 1988.

J. Papa Rao, Zafar, R.S., Sodja, A. A Transcriptional Unit Within a Drosophila Actin Associated 3'UTR, XVIth International Congress of Genetics, Abstracts, #31.12.4, p. 63, 1988.

J. Papa Rao, Zafar, R.S., Sodja, A. Overlapping Transcripts of an Actin Gene in Drosophila melanogaster, 18th Annual Cardiovascular Research Forum, Abstracts, p. 65, 1988.

Jackson, N., Hadden, T.J., Sodja, A. The Actin Genes of Musca domestica, abstract of a poster presented at the 16th Annual NIH-MBRS Symposium, Los Angeles, California, Oct. 13 - 15, 1988.

McMullen, D.M., Winrow, M.A., Sodja, A., Actin Genes in Ascaris suum, 17th Annual NIH-MBRS Symposium, Houston, Texas, Abstract 73, p.24, 1989.

Hadden, T., Kim K., Sodja, A., Molecular Characterization of Actin Genes in Musca domestica, 32nd Annual Drosophila Research Conference, Chicago, Illinois, Abstract 23.149, p. 56, 1991.

Sodja, A., Hadden, T.J., Evolutionary Conservation of a Serum Response Element Between Human and Housefuly Genes, Human Genome '92 International Conference, Nice, France, #132, p. 64, 1992.

Sodja, A., Hadden, T.J., Detailed Characterization of a Putative Muscle-Specific Gene in the Housefly, Musca domestica, 34th Annual Drosophila Research Conference, San Diego, California, Abstract 42A, p. 46, 1993.

Sodja, A., Hadden, T.J., Conservation of Actin Gene Regulatory and Structural Features Between Two Dipterans. 21st Annual NIH-NIGMS Minority Programs Symposium, Abstract 163, p. 53, 1993.

Sodja, A., Hadden, T.J., Analysis of Dipteran Actin Genes Suggests Conservation of Regulatory Elements Between Vertebrates and Invertebrates. Keystone Symposia on Molecular Biology of Muscle Development, J. Cell. Biochem. Supplement 18D, Abstract W 357, p. 522, 1994.

Book Reviews and Other Published Materials

Davidson, N., Fyrberg, E.A., Hershey, N.D., Kindle, K., Robinson, R.R., Sodja, A., Yen, P. 1980. Recombinant DNA studies of DNA Sequence Organization Around Actin and tRNA Genes of Drosophila, In RNA polymerase, tRNA and ribosomes: Their Genetics and Evolution. University of Tokyo, P. 279-295.

NOTE: *This book resulted from an International Symposium on Genetics and Evolution, in Tokyo, 1979 at which this paper was presented.*

Sodja, A., Effect of Tetracycline on Phagocytosis of Polystyrene Spheres by Polymorphonuclear Leucocytes of Guinea Pigs, M.S. Dissertation, 1964.

Sodja, A., Metabolism of Medium Chain Length Fatty Acids in Higher Plants, Ph.D. Dissertation, 1974.

Together with my T.A., Erle Robertson, planned experiments and compiled a lab manual for the Howard Hughes Summer Institute Program on Molecular Biology of the Gene, Winter semester 1991. The actual course offering was in Summer, 1991.

~~Preparation of the Biochemistry laboratory manual for the Howard Hughes Summer~~ Institute Program in Biochemistry, offered in Summer 1994.

Book Review of Biochemistry, 2nd edition, by D. Voet and J.G. Voet, for the International Biodeterioration and Biodegradation, 37:233-235 (1996).

Papers/Posters Presented

Oral Presentations

The National Drosophila Symposium, University of Indiana, Bloomington, Indiana, May 17-19, 1979.

The 6th. Annual College of Biological Sciences Colloquium, Ohio State University, Columbus, Ohio, 6-8 Sept. 1979.

The International Workshop on Molecular Biology of Drosophila melanogaster, Crete, Greece, August 1981 (By invitation).

The 22nd National Conference on Drosophila Genetics, Chicago Ill., April 1981.

Invited speaker: International Congress on Recombinant DNA, Cleveland, Ohio, June, 1981.

Midwestern Drosophila Conference, Allenton Park, Illinois, Oct. 1982 and 1985.

Invited symposium speaker: 149th National Meeting of the AAAS, Detroit, Michigan, 1983, (By invitation).

Invited symposium speaker: 18th FEBS Meetings, Yugoslavia, June 28 - July 3, 1987, (By invitation).

Invited speaker: WSU CLL workshop on Biotechnology, Jan. 30, 1988.

Invited participant: Keystone Symposium: Fundamental Mechanisms of Transcription, Copper Mountain, Colorado, March 28-April 3, 1992.

Invited speaker: Vector Encounter, Case-Western Reserve University, Cleveland, Ohio, June 25-26, 1999.

Poster Presentations

Sodja, A., Hershey, N.D., Conrad, S.E., Davidson, N., Ilgen, C., Carbon, J., ICN-UCLA Symposium on the Molecular Approaches to Eucaryotic Genetic Systems, Park City, Utah, March 1977.

Sodja, A., 1980. Ohio State University Biosciences Colloquia, No. 6, Ohio State University, Ohio, Sept. 1980.

Sodja, A., Zafar, R.S., Cetus UCLA Symposium on the Molecular Biology of Development, Steamboat Springs, Colorado, April 1984.

Sodja, A., Mildner, A.M., Elsenboss, L. UCLA Symposium on Molecular Biology of Muscle Development. Park City, Utah, March 1985.

Winrow, M.A., Martin, P.E., Sodja, A., 13th Annual MBRS Symposium, Miami, Florida, April, 1984.

Elsenboss-Pena, L., Sodja, A., UCLA Symposium on Molecular Entomology, Steamboat Springs, Colorado, April 1986.

Hadden, T. J., Sodja, A., Actin Genes in Two Distantly Related Dipterans, The National Drosophila Meetings, Asilomar, California, April 1986.

Elsenboss-Pena, L., Sodja, A., AWIS, Detroit Science Center, Jan. 31 -Feb. 1, 1987.

Winrow, M.A., Martin, P.E., Sodja, A., UCLA Symposium on Molecular Biology of Invertebrate Development, in Park City Utah, March 15-22, 1987.

Hadden, T.J., Sodja, A., 4th International Congress of Cell Biology, Montreal, Canada, August 14-20, 1988.

J. Papa Rao, Zafar, R.S., Sodja, A., XVIth International Congress of Genetics, Toronto, Canada, August 20-27, 1988.

J. Papa Rao, Zafar, R.S., Sodja, A., 18th Annual Michigan Cardiovascular Research Forum, American Heart Association of Michigan, University of Michigan, Ann Arbor, Michigan, September 15, 1988.

Jackson, N., Hadden, T.J., Sodja, A., 16th Annual NIH-MBRS Symposium Program, Los Angeles, CA, Oct. 13 - 15, 1988.

McMullen, D.M., Winrow, M.A., Sodja, A., 17th Annual NIH-MBRS Symposium, Houston, Texas, Oct. 4-8, 1989.

Hadden, T., Kim K., Sodja, A., 32nd Annual Drosophila Research Conference, Chicago, Illinois, March 20-24, 1991.

Sodja, A., Hadden, T.J., Human Genome '92, 4th International Conference, Nice, France, Oct. 14-17, 1992.

Sodja, A., Hadden, T.J., 34th Annual Dosophila Research Confernce, San Diego, California, Mar. 31-Apr. 4, 1993.

Sodja, A., Hadden, T.J., 21st Annual NIH-NIGMS Minority Programs Symposium, Atlanta, Georgia, Nov. 3-7, 1993.

Sodja, A., Hadden, T.J., Keystone Symposia on Molecular Biology of Muscle Development, Snowbird, Utah, April 11-17, 1994.

NOTE: All of the poster sessions, except for the one presented at Nat'l Drosophila Meetings, have published abstracts listed with full titles and other information under D (Abstracts Published). The oral presentations at the International Congress on Recombinant DNA (Cleveland, Ohio), at the AAAS meetings (Detroit, Mi.) and also at the 18th FEBS meetings have abstracts.

Invited Seminars Presented

University of California, Los Angeles, 1976.
Michigan State University, East Lansing, Michigan, 1976.
University of California, Los Angeles, 1976.
Michigan State University, East Lansing, Michigan, 1976.
Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland, 1977.
Centre of Biochemistry and Molecular Biology, CNRS, Marseille, France, 1977.
Biozentrum, Basel, Switzerland, 1977.
University of Iowa, Iowa City, Iowa, 1977.
University of Amsterdam, Amsterdam, Holland, 1977.
University of Iowa, Iowa City, Iowa, 1977.
Oakland University, Rochester, Michigan, 1979.
Developmental Biology Interest Group, WSU, 1979 and 1983.
University of Michigan, Ann Arbor, 1980.
Case-Western Reserve University, Cleveland, Ohio, 1980.
University of California, Los Angeles, CA, 1980.
Ohio State University, Columbus, Ohio, 1980.
University of Toledo, Toledo, Ohio, 1984.
University of Detroit, Detroit, 1984.
Wayne State University, Biochemistry Department, 1984.
University of Ljubljana, Ljubljana, Yugoslavia, 1984.
Illinois State University, Normal, Illinois, 1985.
Oakland University, Rochester, Michigan, 1986.
Stroh's Brewery Co., Detroit, MI, 1987.
Wayne State University, Dept. of Physiology, 1987.
Heidelberg College, Tiffin, Ohio, 1988.
University of California, Los Angeles, 1993.

Case-Western Reserve University, Cleveland, Ohio, 1995.

Committee Assignments

Community

Judge at the Annual Southeastern Michigan Junior Science Fair and Humanities Symposium held at WSU, 1980 and 1981.

Judge for selecting presentations at this Fair in 1982.

Panel member on the NSF Visiting Professorship for Women, Student Center Bldg., WSU, 1986.

Poster presentation at the Detroit Science Center, Program organized by the Association for Women in Science (AWIS), Detroit Chapter, Jan. 30 -Feb. 1, 1987.

Colloquium speaker at AWIS Workshop on Extramural Funding, Farmington Hills Public Library, March 28, 1987.

As member of the National Association for Women in Science and past president of its Detroit area chapter, I am involved in activities which promote sciences as viable careers for women. Toward achieving its goals our chapter sponsors a number of activities such as a monthly public lecture given by a woman scientist; a weekly workshop at the Detroit Science Center for girl scouts (about 30-60 participants/workshop) and for the third consecutive year we published a calendar featuring women in Science (Nobel Prize laureates and nominees; the 1989 calendar featured scientists inducted into the Michigan Women's Hall of Fame). We have recently developed several fund raising functions: a fund raising banquet featuring Dr. Isabela Lugoski and her Nobel Prize laureate husband; sale of T-shirts and mugs as well as a "corporate letter" to industries interested to support AWIS goals and correct societal scientific illiteracy. The proceeds from these activities enabled us to award 2 scholarships to local high school teachers, enabling them to attend a workshop geared to updating their teaching skills and knowledge in a particular area. Commitments from industries / corporations will enable us in the future to award at least partial scholarships to outstanding female high school/college graduates interested in pursuing a scientific career.

Under the auspices of Detroit Area Pre-college Engineering Program (DAPCEP) together with Dr. M.A. Bednarski from Oakland University, I organized and taught a 10-week lecture/lab course in Genetics and Physiology to about 60 inner city pre-college students in Fall 1989. This course offering, on the campus of University of Detroit-Mercy, was the first "hands-on" laboratory experience for these students. In addition, my involvement in this activity served as a good advertisement for WSU, to which these students were exposed during my lectures and to which they may apply when choosing a

university. It is interesting to note that WSU recently established a middle school, a clear signal of part of the University's mission, especially an inner city such as WSU, to the community. My participation with DAPCEP clearly demonstrates part of that mission and service to the community.

On April 26, 1990, our AWIS Detroit area chapter, together with DAPCEP, sponsored 10 high school students to attend an informative breakfast briefing meeting of the Michigan Technology Council (Dr. R. Thomas, WSU director), Sheraton Southfield, Southfield, MI.

Positions Held in Professional Associations

President of AWIS, Detroit Chapter, June 1988 - June 1990.

Professional Consultations

Consultant to the Biochips Feasibility Study, supported by and submitted to the National Geno Sciences, Inc., 1982.

Enzo Biochem, Inc., 2000.

Journal/Editorial Activities

ad hoc reviewer for Gene (1980-present).

ad hoc reviewer for Proc. Natl. Acad. Sci. USA (1990-present).

reviewer for BioTechniques (1990-present).

reviewer for Insect Molecular Biology (1993-present).

Other Professionally Related Service

Participant in a) NSF, and b) MBRS site visits to WSU, c) visit by the Michigan legislators regarding the recombinant DNA technology/research going on in the Department of Biological Sciences.

Co-organizer of a Symposium at the AAAS meeting May 1983, Detroit, MI.

ad hoc reviewer for NSF, Genetic Biology Program, (1988-present).

ad hoc reviewer for NSF, Systematic Biology, (1989-present).

ad hoc reviewer for Harper & Row Publishing Company, Biology text, 1985, 1986.

ad hoc reviewer for Medical Research Council, London, England, 2000.

External reviewer of a proposal from the Department of Biochemistry, Ohio State University, requesting an endowed chair position for an eminent scholar in protein engineering. The program for the Eminent Scholar positions is sponsored and funded by the Board of Regents of the State of Ohio, 1985.

Member of review panels for predoctoral and postdoctoral programs by the National Research Council (NRC) which reviews such proposals for NRC, Ford Foundation and National Science Foundation Fellowship Programs, 1986-present.

Member on the American Awards Panel for the American Association of University Women, 1986-present.

Research Peer Review Committee member for American Heart Association, Michigan, 1988-1991.

Reviewer for Student Research Study Section, American Heart Association, Michigan, 1990, 1991.

Judge of student posters at the Second Annual Minority Research Programs Day, August, 1999.

#######

Volume 5 f ber 2 February 1978 Nucleic Acids Research

Gene mapping and gene enrichment by the avidin-bi tin interaction: use f cytochrome-c as a polyamine bridge.

Ann Sodja and Norman Davidson

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125, USA

Received 31 October 1977

ABSTRACT

A modification of previously described methods of electron microscopic gene mapping and of gene enrichment based on the avidin-biotin interaction is presented. The modification consists of coupling cytochrome-c instead of pentane diamine to the oxidized 2', 3' terminus of an RNA by Schiff base formation and BH_4^- reduction. The RNA-cytochrome-c conjugate is purified by a simple chromatographic procedure; several biotins are attached to the cytochrome moiety by acylation. The extended arm between biotin and RNA gives efficient electron microscopic gene mapping of DNA:RNA-biotin hybrids with avidin-ferritin and avidin-polymethacylate sphere labels and efficient gene enrichment by buoyant banding of DNA:RNA-biotin:avidin-spheres in CsCl. A 1400 fold enrichment (thus, 25% pure) and c 30% yield of long Drosophila DNA strands with 5S RNA genes is achieved.

INTRODUCTION

The preceding paper (1) describes a method of mapping, with a ferritin label, a short RNA:DNA hybrid region along a single stranded segment f DNA. The essential features of the method are: a) covalent attachment of biotin to the periodate oxidized 3' terminus of the RNA by a diamine bridge, using a simple diamine such as $NH_2(CH_2)_5NH_2$; b) covalent attachment of avidin t the electron opaque label ferritin; c) hybridization of the covalent tRNA-biotin conjugate to a single stranded segment of DNA that contains the coding sequence (gene) for the tRNA; d) electron microscopic mapping of the position of the hybridized tRNA-biotin along the single strand segment of DNA after binding of ferritin-avidin to the biotin. This method gives a moderately satisfactory overall efficiency of gene labeling, and has been used to map the tRNA genes of HeLa mitochondrial DNA (2). We describe here an improved method in which we use a defined polyamine instead of a diamine as the bridge between the 3' end of the RNA and the carboxylic acid biotin. The polyamine used is the protein, cytochrome-c. This bridge is believed to have several advantages: a) it is probably more extended than the pentane diamine; b) several biotins can be attached t ne RNA molecule; c) the

purification step for tRNA-biotin with the pentane diamine bridge involves elution from avidin sepharose at pH 2.5 in 6M guanidine hydrochloride. These fairly drastic conditions are replaced for purification of tRNA-cytr chrome-c-biotin (tRNA-cc-biotin) by hydroxyapatite chromatography at neutral pH, thus diminishing the probability of chemical degradation of the labeled RNA. As a result, presumably, of items a, b, and/or c, the overall efficiency of gene labeling with ferritin in the electron microscope by the new method is an improvement over that achieved previously. d) with the new extended bridge but not with the diamine bridge, avidin attached to poly-methylmethacrylate spheres (3) will label DNA:tRNA-biotin hybrids.

Furthermore, the binding of avidin-spheres by DNA:RNA-biotin molecules forms the basis for a gene enrichment procedure. The polymer spheres used have a density and molecular weight of about 1.25 g/ml and 7.6×10^7 daltons, respectively. The spheres can be used as relatively massive floats to separate DNA:RNA-biotin-avidin-sphere molecules from unhybridized DNA strands by buoyant banding in CsCl, thus accomplishing gene enrichment. Manning, Pellegrini and collaborators have demonstrated this application of the avidin-biotin labeling approach for long RNA molecules, by enriching for the rDNA genes of Drosophila and for the histone genes of the sea urchin (4,5). In these cases, it is appropriate and convenient to attach cytochrome-c-biotin nonspecifically to the RNA by CH_2O crosslinks. In the present communication, we show that 3' terminal labeling of 5S RNA by cytochrome-c can be used for highly efficient gene enrichment of the Drosophila 5S RNA genes.

The basic reaction scheme of the present procedure is:

1) Oxidation of free 2', 3' OH ends of RNA to the dialdehyde with periodate.

2) Schiff base formation of the terminal dialdehyde with the poly-amine, cytochrome-c, at relatively low ionic strength, and stabilization of the compound against dissociation and/or β elimination by BH_4^- reduction.

3) Purification of RNA-cytochrome-c from free RNA and free cytochrome-c by sequential chromatography on carboxymethyl cellulose (CMC) and on hydroxyapatite (HAP).

4) Covalent attachment of several biotin molecules to lysine NH_2 groups of the cytochrome-c by acylation with the N-hydroxy succinimide (NHS) ester of the carboxylic acid biotin.

5) Hybridization of the RNA-cc-biotin to DNA.

6) Labeling with avidin-ferritin or avidin-spheres.

7) Gene mapping by electron microscopy or gene enrichment by banding in CsCl.

MATERIALS AND METHODS

Nucleic Acids. E. coli tRNA's, ϕ80 and ϕ80 psu_3^- DNA's, were btained as described (1). Drosophila (Dm) 4S and 5S RNA and the Drosophila plasmids, pCIT19 and pCIT12, were prepared as previously described (6,7). Unlabeled as well as ^{3}H-labeled Dm DNA's were isolated from Schneider's line 2 tissue culture cells. Cells were labeled by the addition of 1.5 m Ci of ^{3}H-thymidine (Amersham Radiochemical Center, 41 Ci/mmol) to 50 ml of cells grown in suspension with gentle swirling. Cell density at the first addition of label was 1-2 x 10^6 ml; label was added in four equal fractions at 6 hr intervals, and cells grown for another generation (approximately 24 hrs) after the last addition. Cells were harvested by centrifugation at 2400 rpm at 0°C for 5 minutes, lysed by homogenization (10-15 strokes) in 0.5 M Tris base 0.025 M KCl, 5 mM $Mg(Ac)_2$, 0.35 M sucrose, pH 7.6. DNA was prepared from this lysate by the procedure of Manning et al. (8). The specific activities of two separate preparations were 5.4 and 0.84 x 10^5 cpm/µg.

Cytochrome-c. Commercial cytochrome-c (horse heart, type VI, Sigma) is contaminated with RNAase, which has approximately the same molecular weight and charge. RNAase was inactivated by treatment with iodoacetate by a modification of published procedures (9,10). Cytochrome-c (60 mg) was dissolved in .1 ml of 0.2 M NaAc buffer (pH 5.5). An equal weight of iodoacetate was added, the pH readjusted to 5.5 with concentrated NaOH, and the solution diluted to a final volume of 2 ml. The solution was incubated for 1 hr at 55°C and then dialyzed extensively at 0°C against 0.01 M sodium phosphate buffer (NaP), pH 6.8, and lastly against 0.1 M $NaHCO_3/Na_2CO_3$ buffer, pH 9.2.

Preparation and Purification of RNA-Cytochrome-c. tRNA or 5S RNA were heated at 80° for 1-8 min in 1 mM NaAc buffer pH 6.8, cooled, adjusted to 0.1 M NaAc buffer (pH 4.8) and treated with periodate as previously described (1). The amount of RNA used was 0.5 - 1 mg in 0.5 - 1 ml of reaction mixture.

Oxidized RNA was dialyzed against 0.1 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.2) at 0°C. A 10-15 fold molar excess of cytochrome-c was added and the solution

---

ABBREVIATIONS

RNA-cc-biotin, 4S or 5S RNA-cytochrome-c-biotin; HAP, hydroxyapatite; CMC, carboxymethylcellulose; NHS-biotin, N-hydroxysuccinimide ester of biotin; NaP, sodium phosphate buffer (50:50 mono and dibasic sodium phosphate); Dm DNA r RNA, Drosophila melanogaster nucleic acids; EM, electron microscopy

incubated for 1 hr at room temperature. A total of 2 mg $NaBH_4$/1 mg RNA was added in 4 portions over a period of 80 min. The solution was all wed to stand at room temperature for an additional 30 min and the $NaBH_4$ decomposed by addition of 0.1 - 0.2 ml of 4 M NaAc buffer (pH 5.0). The contents were dialyzed at 0°C against 0.01 M NaP buffer, pH 6.8. All of the steps up to and including reduction with $NaBH_4$ were performed in the dark.

For the spectrophotometric determination of concentration, we use molar extinction coefficients of 9.64×10^4 at 410 mμ, 5.4×10^5 and 8.3×10^5 at 260 mμ for cytchrome-c, 4S RNA, and 5S RNA, respectively.

Free cytochrome-c was removed from the reaction mixture by passage over a 3 x 1 cm column of carboxymethyl cellulose (CMC) that had been reequilibrated with 0.01 M NaP buffer. The CMC had been washed with acid and base according to the directions provided by the supplier. The sample was loaded and washed in 0.01 M NaP buffer. Free tRNA and the tRNA-cytochrome-c conjugate come through in the first wash.

Hydroxypatite (HAP, Bio-gel HTP from Bio-Rad) was hydrated by boiling for 10-20 min in 0.01 M NaP, pH 6.8, washed with 0.5 M NaP buffer, and reequilibrated in 0.01 M NaP. For each washing the suspension was swirled gently and allowed to settle for 10-15 minutes before decanting the finer particles. The HAP was packed into a 2 x 0.5 cm column in 0.01 M NaP and the mixture of tRNA and tRNA-cytochrome from the CMC column applied. The column was successively washed with 10 ml volumes of 0.1 M, 0.15 M, 0.3 M and 0.5 M NaP buffer, pH 6.8. Fractions (0.5 - 1 ml) were collected, and assayed by spectrophotometry.

Addition of Biotin. The N-hydroxysuccinimidyl (NHS) ester of ^{14}C-biotin was prepared as described (1). The 1:1 conjugate, tRNA-cytochrome-c, from the HAP column was treated with an approximately 100 fold excess of NHS-^{14}C-biotin under conditions previously described (3). Free biotin was removed by dialysis against 0.01 M NaP and the tRNA-cc-biotin stored at -20°C.

EM Labels. Ferritin-avidin was a gift from L. Angerer (1). Polymethylmethacrylate spheres (a gift from N.D. Hershey) were conjugated to avidin as previously described (3). One of the sphere-avidin preparations was a gift of M. Pellegrini.

Heteroduplex formation and electron microscopy. Heteroduplex formation between Dm plasmids containing 4S or 5S genes with DNA of the vector, Colicin E1, has been described (6,7).

φ80h/φ80 psu_3 heteroduplexes were formed as follows: a solution containing equal amounts of φ80h and φ80 psu_3 bacteriophage was treated with 20 μl

of 0.2 M EDTA (pH 8.0) for 30 min on ice. Complete lysis f the virions and denaturation of DNA was accomplished by addition of 20 µl of 1 N NaOH for 10-15 min at room temperature. The mixture was neutralized with 30 µl f 2.5 M Tris HCl. tRNA-cc-biotin was added and the volume was made to 200 µl with 3X recrystallized formamide (99%, Matheson, Coleman and Bell). The final DNA and tRNA concentrations were 3 µg/ml and 10-30 µg/ml. Hybridization was performed by dialysis of this mixture against 40% 3X recrystallized formamide, 0.1 M Tris, 0.3 M NaCl, 1 mM EDTA, pH 8.0 at 40°C for 40-50 min. Subsequent manipulations were as described (2). The concentration of spheres-avidin in labeling experiments was approximately 100 µg/ml. No removal of excess spheres-avidin was attempted, as an effective procedure to do s is not available.

All electron microscopy and measurements of molecular lengths were done as previously described (2, 6, 7). Single and/or double stranded ϕX 174 DNA (5370 nucleotides or nucleotide pairs, (11)), was used as a length standard.

Preparation of Dm ^{125}I 5S RNA. ^{125}I-5S RNA was prepared essentially according to Orosz and Wetmur (12). The reaction mixture contained, in the order of addition, the following: 10 µl H_2O (double distilled), 3-5 µl Dm 5S rRNA (1.9 mg/ml in 0.01 M NaAc, pH 4.8), 3 µl of 1 M NaAc (pH 5.0), 10 µl ^{125}I (Amersham, carrier free, 100 µCi/ml), and 3 µl of freshly prepared $TlCl_3$ (ICN-K & K Laboratories, 18 mg/10 ml double distilled H_2O). The mixture was incubated at 60° for 20 min in a sealed siliconized 50 µl pipette. The contents were transferred to 1 ml of 0.1 M NaAc buffer (pH 5.0) containing about 50-60 µg of Dm 18S and 28S rRNA, and dialyzed against 0.5 M NaCl, 0.015 M NaH_2PO_4, 2 x 10^{-4} M EDTA, pH 6.0 at 0° (2 x 500 ml) and at 60°C (2 x 500 ml) and at 0°C again until no counts were detected in the dialysate. The ^{125}I-5S RNA preparation was then treated with 50 µg/ml proteinase K (EM Laboratories, Inc.), phenol extracted, and further purified as described (4) or on Cs_2SO_4 gradients. Specific activities obtained in the different preparations ranged from 0.2 - 1 x 10^8 cpm/µg.

Solution Hybridization of ^{125}I-5S RNA to DNA fractions. The contents of 5S genes in the several fractions of Dm DNA for the gene enrichment experiments were carried out by saturation hybridization using excess ^{125}I-5S RNA in solution. DNA solutions were denatured in 0.2 M NaOH, neutralized, and adjusted to the 80% formamide hybridization solution described in the next section. All of the samples contained 10^{-4} M KI in order to reduce background. Typical concentrations f DNA assayed in the respective fractio were 0.075 - 0.5 µg/ml, 13-38 µg/ml, and 26-400 µg/ml, in the enriched, un-

fractionated, and depleted fractions, respectively. Reacti ns were carried out t a rot of 0.06 - 0.6 mol sec/liter. Samples were diluted 10 fold with 2 x SSC and treated at 37° for 1 h ur with RNAase (100 µg/ml RNAase A, 4 units/ml T1 RNAase).

Gene Enrichment Procedure. RNA:DNA hybridization for the gene enrichment experiments was carried out in a high formamide solvent (13) which permits RNA:DNA hybridization but little or no DNA:DNA reassociation. Formamide was 3X recrystallized. DNA in 80% formamide, 2XSSC, was denatured by heating to 80°C for 10 min. A typical hybridization mixture contained 100 µg/ml Dm ^{3}H-DNA, 10 µg/ml Dm 5S RNA-cc-biotin, 150 - 1000 µg/ml Dm 18 + 28S rRNA, all in 2XSSC, 80% formamide at 45°C for 30 min (rot = 6 x 10^{-2} mol sec l^{-1} for the 5S RNA). The sample was dialyzed at 0°C against 0.1 M NaCl, 1 mM Tris, 1 mM EDTA, pH 8.5, and passed over a Sepharose 2B column (19 x 1 cm) to remove excess 5S RNA-cc-biotin. Elution volume has previously been calibrated with Dm ^{3}H-DNA and ^{125}I 5S RNA. The volume of the DNA fraction was reduced to about 100-500 µl by evaporation in a vacuum desiccator. In different experiments 50-150 µl of avidin-spheres (10-15 mg/ml) were added either during or after the evaporation. The solution was adjusted to 1 M NaCl and allowed to stand for 12-16 hours at room temperature or 48 hrs at 0°C. Spheres and DNA bound to spheres were separated from free DNA by banding in CsCl as described (4) except that centrifugation was performed for 48 hrs. The amounts of DNA in the different fractions were determined by ^{3}H counting. DNA was released from the spheres and the RNA hydrolyzed by treatment with 0.2 M NaOH at 100°C for 20 min (14) or at 37°C for 16 hours. The 5S gene content of the several fractions was determined as described above.

RESULTS AND DISCUSSION

Preparation and Purification of RNA-cytochrome-c-biotin. tRNA or 5S RNA was reacted with cytochrome-c as described. The first step in the purification of the reaction product from the starting materials is passage over a CMC column in 0.01 M NaP buffer. Spectrophotometric monitoring showed that neither tRNA nor tRNA-cytrochrome-c binds to the column whereas the free cytrochrome-c does. The crucial step in the purification of RNA-cytochrome-c from unreacted RNA is HAP chromatography. As shown in fig. 1, tRNA elutes from HAP in a 0.15 M NaP wash, whereas RNA-cytochrome-c is eluted by 0.3 M NaP. The absorbance peaks at 410 and 260 mµ show that the material being eluted at 0.3 M NaP is the conjugate with 1:1 molar rati .

Several comments sh uld be made about the procedure. By using a 10-15 fold excess of cytochr me-c to RNA we decrease the probability of forming

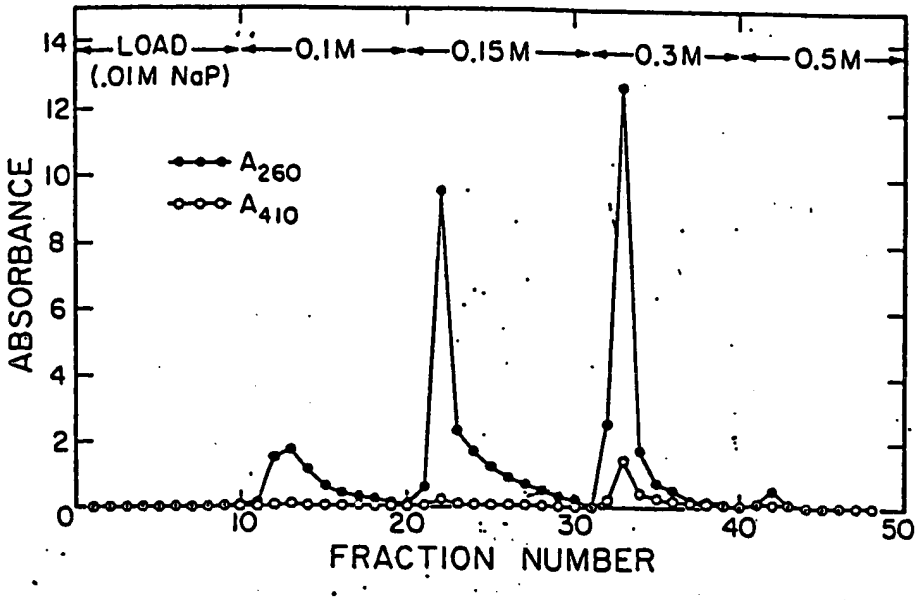


Fig. 1. Elution profile of tRNA-cytochrome-c from a HAP column. Batchwise elution of the reaction components was performed as described in Meth ds. tRNA and cytochrome-c concentrations were measured by absorbance at 260 mμ and 410 mμ, respectively. The column was loaded in 0.01 M NaP, pH 6.8. No A_{260} or A_{410} was observed in the washes with this solvent. The concentrations of tRNA and of cytochrome-c in the elution peaks were calculated as (tRNA) = $(A_{260}/5.4 \times 10^5)$ mole liter^{-1} and (cyt-c) = $(A_{410}/9.6 \times 10^4)$ mole liter^{-1}. The total amounts of tRNA and cytochrome-c in the peak eluting at 0.3 M NaP above are 35 and 34 nmoles, respectively.

molecules such as $(RNA)_2$-cytochrome-c. The elution profiles vary somewhat with the batch of HAP used and with its preparation. Our results have been reasonably reproducible when the HAP is freshly prepared for each experiment as described in Materials and Methods. Nevertheless it is necessary t monitor the absorbance profiles for each new purification, and to make slight adjustments of the salt concentrations of the elution buffers accordingly.

The final results of all experiments on the preparation of RNA-cytochrome-c and of RNA-cc-biotin are given in table I. The yields of RNA-cytochrome-c in some of the earlier experiments were poor. We now suspect that the Drosophila tRNA used was charged with amino acids, but we failed to include a deacylation step, hence the poor yield in these experiments. The best yields with deacylated E. coli tRNA are approximately 50%. For 5S RNA it proved necessary to pretreat the cytochrome with iodoacetate to inactivate RNAase, otherwise the 5S RNA was extensively degraded. We believe that this step is advisable for tRNA preparations also, but it was n t done in the experiments of table I. Heating the RNA sample to 80° before coupling appears to improve the yield, possibly because it causes dissociation of aggregates f rmed during lyophilization or ethanol precipitation.

Table I

Effect of Different Treatments on Yields of RNA-cytochrome-c-biotin

RNA	Treatment	% Yield (1:1 RNA/cyto-c)	Molar excess of biotin/RNA	Number of biotins/RNA-cyto-c
E. coli tRNA	no heat step	28.7	50	5
E. coli tRNA	no heat step	12.7	33	3
E. coli tRNA	80°C, 1 min	46	190	7
E. coli tRNA	80°C, 1 min	48	100	7
[a]Dm tRNA	80°C, 1 min	11.2	240	9
Dm tRNA	80°C, 1 min	18.7	100	10
[b]E. coli 5S rRNA	no heat step	30	100	7
E. coli 5S rRNA	70°C, 10 min	55	100	5
Dm 5SrRNA	70°C, 10 min	56	100	9
Dm 5S rRNA	70°C, 10 min	43	100	5

[a]As mentioned in the text, Dm tRNA was not deacylated and hence lower yields.

[b]Results with 5S rRNA are those where cytochrome-c was pretreated with iodoacetate as described in Methods. Initially, when the iodoacetate step was omitted, the yields of the final product were low (0-5%).

As shown in table I, a molar excess of 50-100 fold of NHS-biotin to tRNA-cytochrome-c was used in order to obtain a final product with 3-10 biotins per cytochrome. Control experiments with unconjugated RNA gave undetectable binding of biotin after treatment with NHS-biotin and dialysis.

Cytrochrome-c is positively charged and does not elute from the negatively charged resin, CMC, until the NaCl concentration is raised to approximately 5 M, whereas both tRNA and tRNA-cytochrome-c are negatively charged and d not bind to the column even in 0.01 M NaP. The crucial step in the purification is the HAP chromatography step. Unligated cytrochrome-c elutes with approximately 0.5 M NaP, free tRNA with about 0.15 M NaP, and the 1 to 1 RNA-cytochrome conjugate at 0.3 M NaP. Several other separation methods were tried without success, including DEAE chromatography both in denaturing and nondenaturing conditions, CsCl centrifugation and gel filtration.

EM Mapping: The ϕ80psu$_3^-$/ϕ80 heteroduplex. This heteroduplex is a convenient test system for tRNA mapping techniques. As shown in previous studies (15, 16) and sketched in fig. 2, the heter duplex loop consists of a 3100 nucleotide segment of E. c li DNA and a 2100 nucleotide single-strand

segment of ϕ80 DNA. The substitution begins at the att site of ϕ80 DNA. The E. coli single strand segment contains 1 $tRNA^{tyr}$ gene at a p sition 1100 nucle tides from the att junction. Electron micr graphs of two heteroduplexes labele with spheres-avidin are shown in fig. 2. Micrographs (not shown) of hetero-duplexes labeled with ferritin-avidin are comparable in appearance to th se obtained by other methods (2, 15). A histogram of the spheres positions is given in Fig. 3. The results are in accordance with previous mapping data.

A considerable background of free spheres is evident in the micr graph. We have not found a procedure for separating unbound spheres from those attached to DNA, comparable to the sodium iothalamate buoyant banding procedure (1) that can be used to separate free ferritin from ferritin

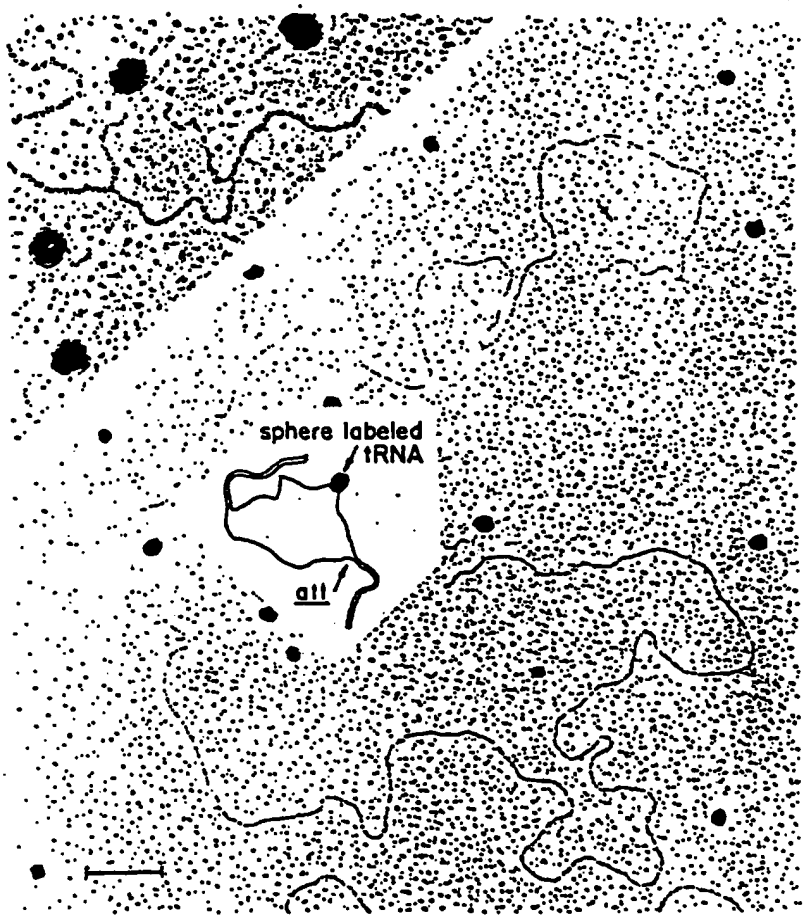


Fig. 2. Electron micrographs of sphere labeled tRNA genes on ϕ80/ϕ80psu$_3^-$ heteroduplex. One complete molecule is shown, with an inset sketch f the heteroduplex loop. The att site and the fork at the other end of the sub-stitution loop are 23.8 and 19.3 kb from the left and right ends of the heteroduplex, respectively (15); theref re they are readily distinguished. An inset photograph of the heteroduplex loop of a second molecule is shown; the magnification is 2X that of the other photo. Bar = 1 kb.

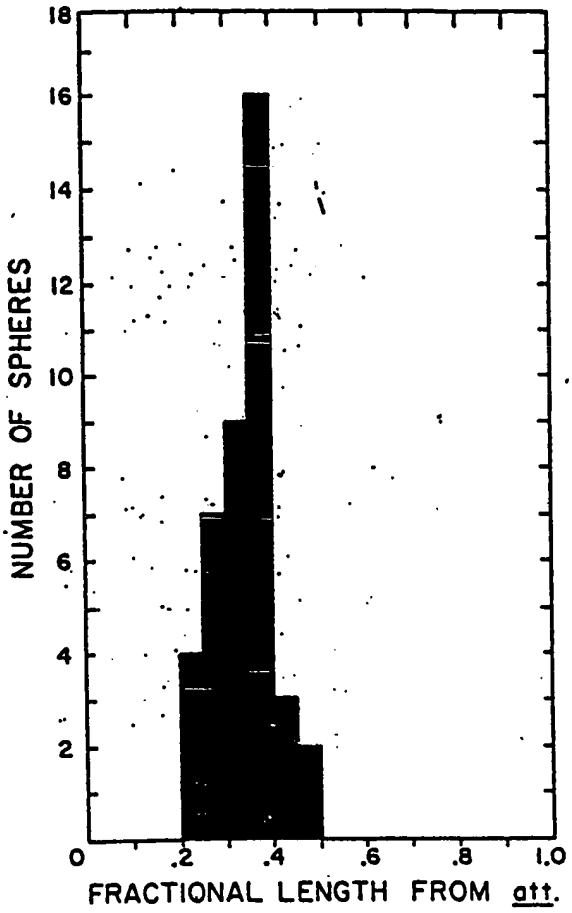


Fig. 3. Histogram of map position of $tRNA^{tyr}$ gene on the single strand of bacterial DNA of the $\phi80/\phi80$ psu_3^- heteroduplex. The horizontal coordinate is the fractional distance from the att site to the label. The measured position of the label is 1100 (± 66) nucleotides from the att site.

labeled heteroduplexes. It may be noted however, that the spheres in the background seem to avoid the DNA strands.

By counting random fields we estimate labeling efficiencies per gene of 50-60% for both ferritin-avidin and the sphere-avidin labels using the cytochrome-c bridge. In experiments with the pentane diamine linker, the labeling efficiency with spheres was negligibly small (N.D. Hershey, personal communication). Presumably the greater length of the cytochrome-c linker is responsible for the efficient labeling in the present method. Nevertheless it should be noted that the efficiency of labeling either with spheres-avidin or ferritin-avidin is at best about 60%. The reason why a figure closer to a 100% is

not achieved is not known.

EM Mapping. Drosophila 4S and 5S genes. Studies from this laborat ry on the mapping of 4S RNA genes on a 9.3 kb segment f Drosophila melan gaster (Dm) DNA c ntained on the recombinant DNA plasmid pCIT12 have been rep rted (7). The mapping data were obtained by EM mapping, using the technique described in detail in the present paper with a ferritin-avidin label, and by restriction endonuclease - hybridization mapping.

Electron micrographs from additional mapping experiments with the avidin-sphere label on the plasmid pCIT12 are shown in fig. 4. A histogram of the observed positions of the tRNA genes on the Dm insert is shown in fig. 5. In the previous study as well as in the present one labeled sites (genes) were found at the 3 positions 1.38 ± 11, 4.59 ± 0.17 and 8.38 ± 0.26 kb from the defined left end of the Dm insert. An additional gene mapping in the position 5.6 - 6.2 kb was found by restriction endonuclease mapping but was not detected at an appreciable frequency in the ferritin-avidin studies. The histogram in fig. 5 shows that this gene, mapped at 5.89 ± 0.36 kb was labeled at a frequency lower than that for the other genes but at a clearly detectable level in the present sphere-avidin studies. We do not know at present whether this improved labeling efficiency is due to the fact that the avidin spheres contain more avidins per label (8-10) than do the ferritin labels (1-2), or due to some other unknown factor.

Ferritin-avidin mapping studies, using the present techniques, on the recombinant plasmid pCIT9 with an insert carrying 3 Dm 5S RNA genes have been reported (6). Drosophila 5S genes are tandemly repeated with a regular spacing of about 380 nucleotides. Our labels -- ferritin-avidin or spheres-avidin-- are multivalent, in that they contain several biotin binding sites per label. Many tangled structures were seen because one label was attached to several hybridized RNA-biotin molecules along a DNA strand. Such molecules cannot be accurately analyzed; nevertheless we have estimated that the overall efficiency of labeling per gene is 40-50% in the various experiments.

As an overall evaluation then, the present method gives 40-60% labeling efficiency in practical problems. Resolution is probably limited by the diameters of the labels - about 200 Å for ferritin-avidin and 600 Å for spheres-avidin. At present, difficulties are encountered with closely spaced genes because of the multivalent character of the labels. Further work is needed to develop a ferritin-avidin conjugate with only one avidin per ferritin and with a high efficiency of labeling, so that closely spaced multiple genes can be mapped.

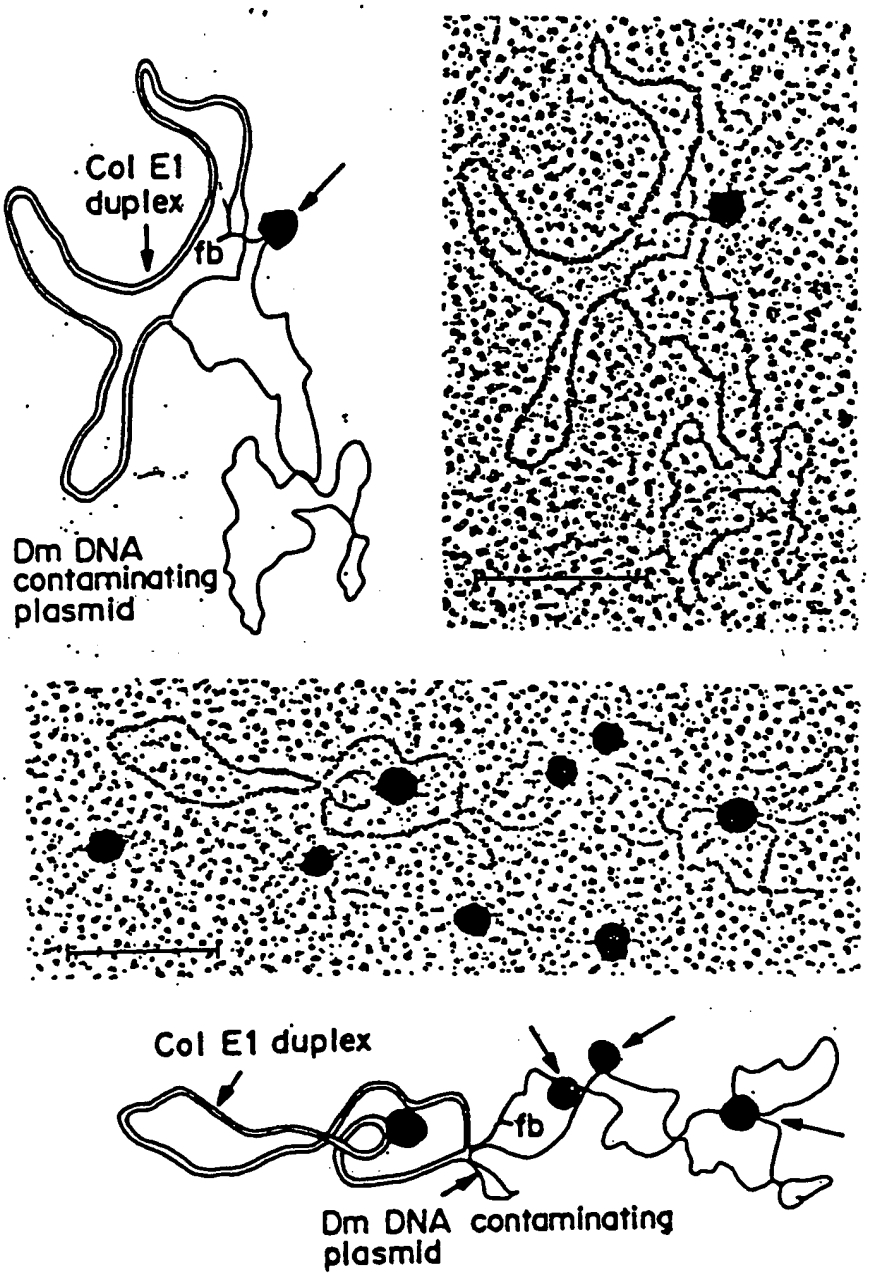


Fig. 4. Electron micrographs of sphere labeled tRNA genes on a single strand of the recombinant plamsid pCIT12. For a full explanation of the structures, see (7). Briefly, the molecules shown are heteroduplexes of pCIT12, which is ColE1 fused to a 9 kb Dm insert, with a second contaminant plasmid consisting of ColE1 fused to another short Dm insert. A secondary structure feature, fb, on the 9 kb single strand serves to orient that strand. The single tRNA gene labeled in the upper micrograph is at the position 8.38 ± 0.26 in fig. 5; the three sphere labels in the lower micrograph are at the positions 1.38 ± 0.11, 5.80 ± 0.36 and 8.38 ± 0.26 in fig. 5. In addition there is a sphere nonspecifically attached t the C 1E1 duplex. These are rare. Bars = 1 kb.

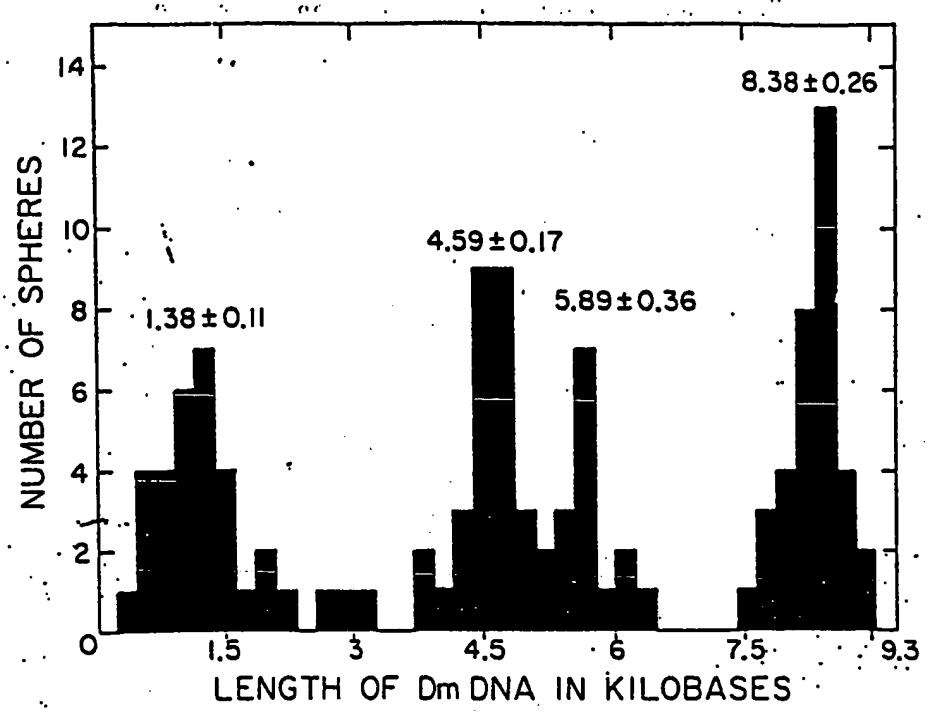


Fig. 5. Histogram of the distribution of Dm tRNA gene positions on the pCIT12 plasmid. The measurements were done as described (7). In addition t the 3 genes found in the previous ferritin mapping study, a 4th gene at 5.89 kb was labeled here, although to a lesser extent. Its position is in agreement with that determined for the 4th gene by the restriction endonuclease mapping.

Gene Enrichment. In these experiments, high molecular weight Drosophila DNA is incubated with 5S RNA-cc-biotin. Hybridization is carried out in a high formamide solvent under conditions where RNA:DNA association is favored over DNA:DNA association (13). Those DNA strands which hybridize to the 5S RNA are separated from all other strands by reaction with avidin-spheres and buoyant banding. These experiments are basically identical to those previously described for the enrichment of the rRNA genes of Drosophila (4), except that, as appropriate for the short length of the 5S RNA, we use a terminal cytochrome-c bridge between the RNA and the biotin instead of random crosslinking of cytochrome-biotin to the RNA by CH_2O.

There are 160 5S RNA genes of Drosophila (17). They are tandemly repeated with a repeat spacing of about 380 nucleotides. They may occur as two clusters of approximately 80 genes each or there may be a single cluster of length 63.4 kb containing all of the genes, as suggested by restriction digest studies of the chromosomal DNA (17, 18). All told, the genes plus spacers constitute 1.73×10^{-4} of the haploid Dm genome (1.8×10^8 base

pairs). In the present experiments the bulk of the single strands of Dm DNA ranged in length from 10-100 kb. Thus the strands carrying 5S genes would have 25 or more 5S RNA-cc-biotin molecules hybridized. (There is every theoretical reason t expect that the efficiency of RNA:DNA hybridization is almost 100%.) For a strand of length 10 kb with one sphere attached the weight fraction of sphere mass is 0.96. If several spheres are attached per 10 kb the weight fraction is still closer to 1. Thus by the rules formulated by Pellegrini et al. (5) the buoyant density of the DNA sphere complex would be close to that, 1.25 g/ml, of uncomplexed spheres. The efficiency of labeling in the electron microscope experiments was 50% per gene. Just how many avidin-spheres would be bound to a 10 kb strand carrying 25 hybridized 5S RNA-cc-biotin molecules is uncertain because we do not know how many of these biotins would bind to a single avidin-sphere rather than to different avidin-spheres.

The results of several gene enrichment experiments are presented in table II. In all three experiments, almost all (greater than 88%) of the 5S genes were recovered in the enriched fraction. In the first experiment, about 1.5×10^{-2} of the total DNA was in the enriched fraction whereas in the second and third experiments only about 6.5×10^{-4} of the DNA was in the

Table II

Results of Enrichment for 5S rDNA from Total Drosophila DNA

	% Total DNA in		% 5S rDNA in		Enrichment Factor
	Enriched	Depleted	Enriched	Depleted	
Theoretical Experiment	0.0173	99.923	100	0	5780
1	1.48	98.52	99.9	not detected	67
2	0.067	99.933	91.5	8.5	1351
3	0.063	99.937	88.8	9.8	1411

The volume of the DNA after the Sepharose 2B step is usually 7-10 ml. The salt concentration is 0.1 M. This volume is reduced by evaporation by a factor of 10 and the salt concentration raised to 1.0 M for sphere labeling under conditions of minimal electrostatic interaction between the positive avidins on the spheres and the negative DNA. In the first experiment, the spheres were added before the evaporation step. Perhaps this caused more quasi-irreversible electrostatic binding between spheres and DNA. In experiments 2 and 3, spheres were added after evaporation. In the 3rd experiment, labeling was done at 0° for 48 hr instead of at room temperature for 16-24 hours.

enriched fraction. Theref re in the first experiment the 5S DNA after one cycle of enrichment was about 1% pure, whereas in the second and third experi ments it was about 25% pure, In control experiments where no RNA was added approximately 3.4×10^{-4} of the DNA was found in the sphere band. Therefore of the total of about 6.5×10^{-4} DNA in experiments 2 and 3, about 50% may be attributed to general binding or trapping of DNA by the spheres, 25% to specific gene enrichment, and 25% to other causes, possibly formation of networks or partially duplex DNA with a strand bearing a 5S gene (see below).

The two major technical differences between experiment 1 and the more successful experiments 2 and 3 were:

1) In the latter experiments, the excess of unlabeled (no cytochrome-biotin) rRNA (which included 5.8S rRNA as well as 18 and 26S) added was 100 times the amount of 5S RNA, whereas in the first experiment there was only a 15 fold excess. Whereas 5S genes plus spacers make up 1.7×10^{-4} of Dm DNA, rRNA genes plus spacers constitute 6.4×10^{-3}; therefore it is necessary to completely compete out hybridization of any biotin labeled rRNA fragments present as contaminants in the 5S preparation with the cold rRNA. In their filter hybridization experiments, Tartof and Perry (19) found that it was necessary to use a 100 fold excess of unlabeled rRNA in order to accurately assay for the number of 5S RNA genes.

2) As explained in a footnote to table II, there was a difference in a concentration step of the sphere-avidin-DNA mixture between experiments 1 and experiments 2 and 3 which may have decreased the amount of non-specific binding in the latter.

A small fraction of the sphere band from the CsCl gradient was directly diluted into formamide solution and spread for electron microscopy. The DNA structures observed were of the following types: a) Single strands with one or many spheres. Strands with many spheres were tangled and condensed around the spheres, as expected in view of the close spacing of the genes and the several avidins attached to each sphere. b) Single strands with n spheres attached. These were presumably released from the spheres by breakage. c) Molecules that were partially duplex, due to some DNA:DNA reassociation. d) Networks of single strands, perhaps due to tangling of the long strands in the high salt medium. Factors (c) and (d) may contribute to the amount of non-coding strands in the enriched fraction. The DNA strands observed had about the same length distribution as the input DNA, showing that the gene enrichment procedure does not cause much chain breakage.

Further discussion. In several test systems, the efficiency of labeling with the cytochrome-c-biotin attached to the 3' terminus of 4S and 5S RNA was 50% with either avidin-spheres or ferritin-avidin. The efficiency of labeling by spheres-avidin for cytochrome-bi tin randomly cr sslinked to RNA with CH_2O is reported to be sustantially lower than this figure (4). The present method could be applied to genes for long RNA's as well as for short ones. For long RNA, it would be advantageous to degrade the RNA to a length of 100-400 nucle - tides, and expose new 2', 3' OH ends with alkaline phosphatase before coupling to cytochrome-c. Thus one would provide several cytochrome-biotin affinity labels per gene. In general then, the present technique appears to be a very useful addition to methods of gene enrichment and electron microscope gene mapping.

ACKNOWLEDGMENTS: We would like to thank Dr. Maria Pellegrini for helpful discussions on the enrichment experiments. A.S. was the recipient of a fellowship from the California Section of the American Cancer Society. This research has been supported by grant GM 10991 from the United States Public Health Service.

REFERENCES

1. Broker, T.R., Angerer, L.M., Yen, P., Hershey, N.D., and Davidson, N. (1978) Nucleic Acids Res., preceding paper.
2. Angerer, L., Davidson, N., Murphy, W., Lynch, D., and Attardi, G. (1976) Cell 9, 81-90.
3. Manning, J.E., Hershey, N.D., Broker, T.R., Pellegrini, M., Mitchell, H.K. and Davidson, N. (1975) Chromosoma 53, 107-117.
4. Manning, J., Pellegrini, M., and Davidson, N. (1977) Biochemistry 16, 1364-1370.
5. Pellegrini, M., Holmes, D.S. and Manning, J. (1977) Nucleic Acids Res., 4, 2961-2974.
6. Hershey, N.D., Conrad, S.E., Sodja, A., Cohen, M., Davidson, N., Ilgen, C., and Carbon, J. (1977) Cell 11, 585-598.
7. Yen, P.H., Sodja, A., Cohen, M., Conrad, S.E. Wu, M., Davidson, N., and Ilgen, C. (1977) Cell 11, 763-777.
8. Manning, J.E., Schmid, C.W., and Davidson, N. (1975) Cell 4, 141-155.
9. Gundlach, H.G., Stein, W.H., and Moore, S. (1959) J. Biol. Chem. 234, 1754-1760.
10. Zimmerman, S.B., and Sandeen, G. (1965) Analyt. Biochem. 14, 269-277.
11. Barrel, B.G., Air, G.M., and Hutchison, C.A. III. (1976) Nature 264, 34-41.
12. Orosz, J.M., and Wetmur, J.G. (1974) Biochem. 13, 5467-5473.
13. Casey, J., Davidson, N. (1977) Nucleic Acids Res. 4, 1539-1552.
14. Kotchetkov, N.K., Budovskii, E.I. (eds) (1972) in Organic Chemistry of Nucleic Acids, Part B, p. 493 Plenum Press, London and New York.
15. Wu, M., and Davidson, N. (1973) J. Mol. Bi l. 78, 1-21.
16. Miller, R.C., Besmer, P., Khorana, H.G., Fiandt, M., and Szybalski, W. (1971) J. Mol. Biol. 56, 363-368.
17. Pr curier, J.D., and Tartof, K.D. (1976) Nature 263, 255-257.

18. Artavania-Tsakonas, S., Schedl, P., Tschudi, C., Pirrotta, V., Steward, R., and Gehring, W.J. (1977) Cell, submitted for publication.
19. Tartof, K.D., and Perry, R.P. (1970) J. Mol. Biol. 51, 171-183.

Volume 5 Number 2 February 1978 Nucleic Acids Res arch

Electron microscopic visualization of tRNA genes with ferritin-avidin: biotin labels

Thomas R. Broker*, Lynne M. Angerer, Pauline H. Yen, N. Davis Hershey and Norman Davidson

Department of Chemistry, California Institute of Technology, Pasadena, CA 91125, and
*Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA

Received 31 October 1977

ABSTRACT

A method is described for indirect electron microscopic visualization and mapping of tRNA and other short transcripts hybridized to DNA. This method depends upon the attachment of the electron-dense protein ferritin to the RNA, the binding being mediated by the remarkably strong association of the egg white protein avidin with biotin. Biotin is covalently attached to the 3' end of tRNA using an $NH_2(CH_2)_5NH_2$ bridge. The tRNA-biotin adduct is hybridized to complementary DNA sequences present in a single stranded non-homology loop of a DNA:DNA heteroduplex. Avidin, covalently crosslinked to ferritin, is mixed with the heteroduplex and becomes bound to the hybridized tRNA-biotin. Observation of the DNA:RNA-biotin:avidin-ferritin complex by electron microscopy specifically and accurately reveals the position of the tRNA gene, with a frequency of labeling of approximately 50%.

INTRODUCTION

Electron microscopy has been used to study the organization of genes on chromosomes: (a) by analyzing substitution, deletion and insertion loops in heteroduplex structures prepared between DNA from related genomes (c.f., 1): (b) through cross-annealing identified segments of DNA (such as those present on transducing phages and bacterial F' episomes)(c.f., 2), and (c) by hybridizing purified RNA to complementary sequences in single-stranded (c.f., 3) or double-stranded DNA (c.f., 4). The success of these methods depends upon the existence of sufficiently long duplex regions for reliable discrimination between single and double strands. Methods of indirect visualization of short RNA molecules hybridized to DNA using single strand specific labels such as the T4 gene 32 protein (5) or the E. coli binding protein (6) are difficult to apply to very short RNA:DNA hybrid regions. Wu and Davidson

ABBREVIATIONS

NHS - N-hydroxysuccinimide; DMF - dimethylformamide; DMSO - dimethyl sulfoxide; DTT.- dithiothreitol; GuHCl - guanidine hydrochloride; NaP - sodium phosphate buffer; $NaBH_4$ - sodium borohydride.

(7) developed a labeling method using the electron-opaque protein ferritin covalently attached to the tRNA prior to the hybridization step. This technique has proven to be technically demanding.

We have developed an alternative method for attaching ferritin to RNA in an RNA:DNA hybrid. The RNA is covalently attached to the small molecule biotin and is then hybridized to the DNA. The protein avidin is covalently coupled to ferritin. The ferritin-avidin conjugate is then bound to the biotin-RNA:DNA hybrid by means of the strong non-covalent interaction between avidin and biotin.

In the present report we describe the chemical coupling and purification procedures in detail and our studies of the efficiency of electron microscopic mapping, using as a test system, the $tRNA^{tyr}$ gene on the $\phi 80\ psu_3^-/\phi 80$ heteroduplex. The method gives reproducible results with gene labeling efficiencies near 50%. The tRNA genes of HeLa cell mitochondrial DNA have been mapped by this method (8). A further development of the same basic technique is described in the accompanying paper (9).

An alternative application of RNA-biotin:avidin technology for mapping genes has been described in a previous publication from this laboratory (10). Drosophila ribosomal RNA was coupled to biotin by a different method than the one described here and hybridized in situ to salivary gland chromosomes. This preparation was then treated with avidin coupled to polymethacrylate spheres; the hybrids with spheres attached could be visualized in the scanning electron microscope.

RATIONALE

The overall reaction scheme is illustrated in Fig. 1. The 2',3'-cis hydroxyl terminus of tRNA is oxidized by periodate to the dialdehyde and coupled to one of the amino groups of 1,5-diaminopentane by Schiff base formation and subsequent $NaBH_4$ reduction. Biotin is attached to the remaining amino group of the diamine by acylation with the NHS ester of biotin. The tRNA-biotin conjugate is hybridized to DNA containing the complementary gene sequence. Meanwhile, reactive bromoacetate groups are attached to ferritin and reactive thioacetate groups to avidin by NHS acylation reactions. These groups mediate the crosslinking of ferritin to avidin. The resulting conjugates are used to label the tRNA-biotin:DNA hybrid.

EXPERIMENTAL PROCEDURES

Commercial Materials: A list of purchased materials and their suppliers follows: E. coli tRNA, ^{14}C-cysteine, ^{14}C-N-ethyl maleimide, GuHCl and sucrose, (Schwarz-Mann); ^{3}H-E. coli tRNA, (Miles); avidin and biotin, (Sigma); ^{14}C-

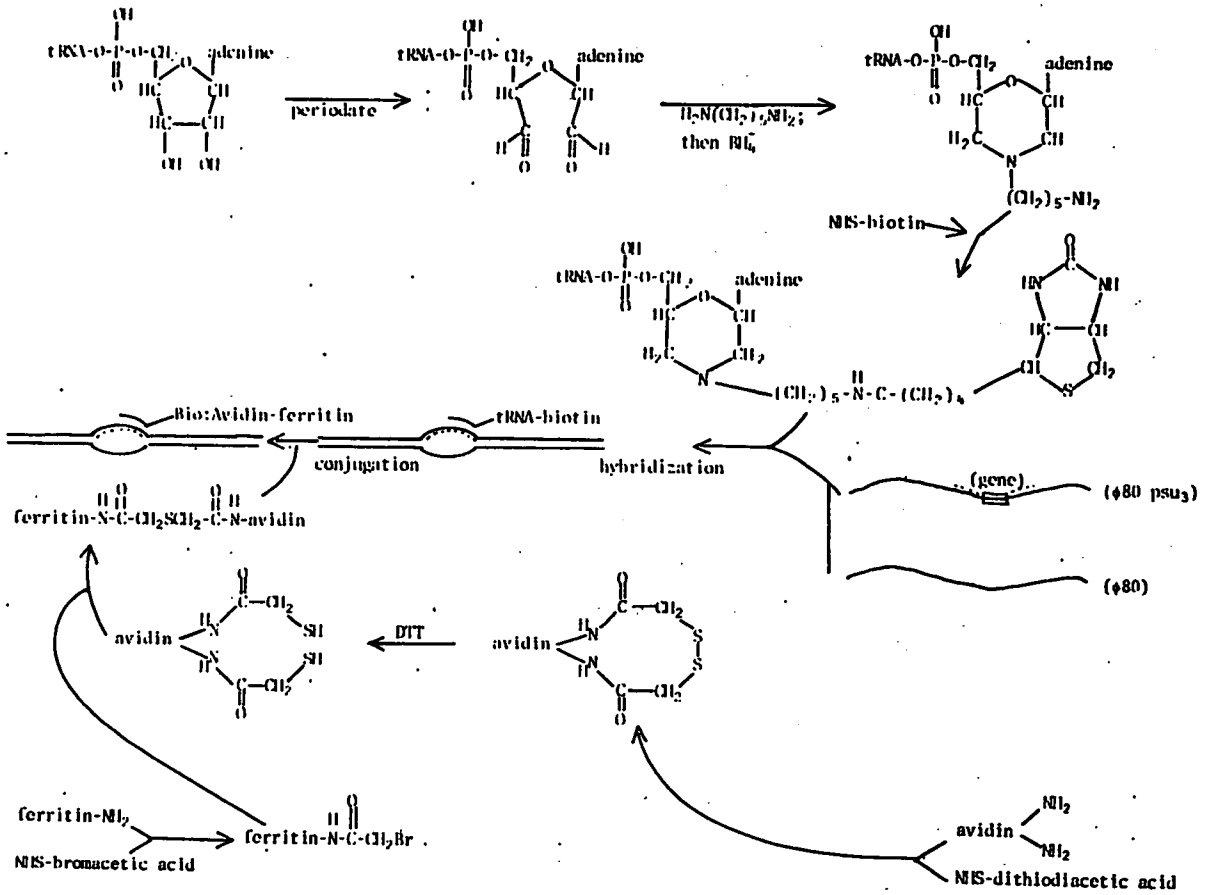


Fig. 1. Overall reaction scheme for covalent attachment of biotin to RNA, avidin to ferritin, and for gene mapping.

biotin, ^3H-$NaBH_4$ (Amersham-Searle); dithiodiacetic acid, NHS, diaminopentane, (Aldrich); dicyclohexylcarbodiimide (MCB); $NaBH_4$ (Metal Hydrides, Inc.); Sepharose 2B and 4B (Pharmacia); DEAE-cellulose (BioRad); DMF, sodium iothalamate (Mallinckrodt); ethyl acetate (Baker).

Buffers: The buffers used in some of these experiments are indicated by the following abbreviations: 0.1 M NaCl, 0.01 M NaP, 0.001 M EDTA, pH 7.5 (1XSPE); 1M NaCl, 0.01 M NaP, pH 7.0 (HSB); 1M NaCl, 2M urea, 0.01 M NaP, pH 7.0 (HSUB). All phosphate buffers were prepared from NaH_2PO_4 with the pH adjusted with NaOH.

Specification and Assays: ^3H-tRNA (25,000 daltons MW; 1 mg/ml = 25 A_{260}) was monitored by optical density and by scintillation counting. Avidin (65,000 daltons MW; 1 mg/ml = 1.54 A_{282} (11)) was assayed by optical density and by its irreversible association with ^{14}C-biotin during exhaustive dialysis, sedimentation or chromatography (12, 13). Binding of ^{14}C-biotin is complete within 10 min when a 2- to 5-fold excess is added to avidin. One mg of avidin corresponds to 15.4 nmoles of the tetrameric protein, which bind 62 nmoles (15.1 µg) of biotin (11). Avidin conjugated to Sepharose or to ferritin probably binds less biotin, which makes assays only approximate. Heitzmann and Richards (14) obtained preparations of gluteraldehyde crosslinked ferritin-avidin with yields of biotin binding of approximately 50%. Holoferritin (900,000 daltons MW; 1 mg/ml = 14.4 A_{280} or 1.54 A_{440} (7, 15)) was assayed by optical density.

NHS-biotin: The NHS ester of biotin was prepared by the method of Becker, Wilchek, and Katchalsky (16). To 12.4 ml of DMF containing 1 g of biotin and 0.475 g of NHS was added 0.85 g of dicyclohexylcarbodiimide. After 15 hr at 25°C, the reaction mixture was chilled to -20°C for 4.5 hr and the dicyclohexylurea precipitate removed by centrifugation. The supernatant was evaporated to dryness in a Buchler vacuum evaporator. The residue was washed with ethanol, dried and then recrystallized from isopropanol. The yield of NHS-biotin was 64%; its m.p. was 198-200°C. NHS-^{14}C-biotin was synthesized similarly by incubating ^{14}C-biotin (50 µC), biotin (2.4 mg), NHS (1.1 mg) and dicyclohexylcarbodiimide (2.1 mg) in 50 µl DMF for 5.5 hr at 25°C. The resulting solution of *ca.* 0.2 M ^{14}C-NHS-biotin (Specific activity = 1.4 x 10^7 cpm/µmole) was used without further purification. An alternate synthesis of NHS-biotin has been described (17).

NHS-dithiodiacetic acid: To a stirred solution of 1.82 g dithiodiacetic acid (0.01 mole) and 2.3 g NHS (0.02 mole) in 100 ml ethyl acetate was added 4.12 g dicyclohexylcarbodiimide (0.02 mole). The mixture was stirred at room

temperature for 2.5 hr. Dicyclohexylurea was removed by filtration and washed with 40 ml ethyl acetate. The combined filtrates were subjected to rotary evaporation at reduced pressure to yield a dark yellow oil. Several recrystallizations from methanol yielded white needles of the NHS ester with m.p. 131.5 - 135°C.

NHS-bromoacetic acid: The preparation of this ester has been described previously (18).

Synthesis of tRNA-biotin: E. coli K12 tRNA at concentrations ranging from 600 µg/ml to 10 mg/ml was incubated for 90 min at 37°C in 2M Tris, pH 8.2 to insure deacylation (19) and was then dialyzed against 0.05 M to 0.1 M sodium acetate, pH 4.7. One tenth volume of 1 M $NaIO_4$ freshly dissolved in water was added. After oxidation for 1 hr at 20°C in the dark, the tRNA was dialyzed at 4°C in the dark sequentially against 2 changes of 0.05 M sodium acetate, pH 5.1, 0.1 M NaCl, and two changes of 0.3 M sodium borate, pH 9.0 - 9.3 $\pm$ 0.1 M NaCl. This solution was made 0.4 M in 1,5-diaminopentane, using a stock of the diamine that had been preadjusted to pH 9.3, and was then incubated for 45-90 min at 20°C in the dark. The resulting Schiff base was reduced with $NaBH_4$ using unlabeled or ^3H-labeled reagent: (i) unlabeled $NaBH_4$, freshly dissolved in water, was added four times at 30 min intervals, resulting in increments of 0.025 M to 0.1 M BH_4^- and incubation was continued for a total of 3 hr at 20°C; (ii) ^3H-$NaBH_4$ (1.28 M and 490 µC/µmole in 1 N NaOH) was added to the Schiff base in 15X molar excess and incubated for 1.5 hr at 0°C, then 2 hr at 20°C. The reduction was then driven to completion by the further addition of unlabeled $NaBH_4$ as described above. Residual $NaBH_4$ was quenched by adjustment to pH 5 - 5.5 with 4M sodium acetate, pH 5. The tRNA-amine was dialyzed extensively first against 0.1 M NaCl, 0.01 M- 0.05 M NaP, pH 6.8, 0.001 M EDTA and then against 40% DMF, 0.03 M NaCl, 0.05 M NaP, pH 6.8. NHS-biotin was dissolved in DMF and added to the tRNA-amine (0.5-5 mg/ml) to a final concentration of 20 mM NHS-biotin and about 50% DMF. The reaction was carried out for 12-16 hr at 20°C. Excess NHS-biotin was removed by dialysis against 40% DMF, 0.15 M NaCl, 0.05 M NaP, pH 7.0 and then against 1XSPE. Some preparations of tRNA-biotin were passed through G-25 Sephadex to remove any residual free biotin. The RNA in the excluded volume was precipitated in 70% ethanol for 24 hrs at -20°C and then redissolved in HSB in preparation for chromatography on avidin-Sepharose.

Preparation of Avidin-Sepharose: Avidin was coupled to Sepharose by a procedure similar to those described previously (20, 21, 22). Sepharose 4B (30 µm-200 µm beads) was washed free of sodium azide and was deaerated under

vacuum. 20 ml of Sepharose slurry was suspended in 20 ml water and adjusted to pH 11 with NaOH. 2-4 gm cyanogen bromide, dissolved in 1-2 ml dioxane, was added dropwise over a 3 min period to the Sepharose with gentle mixing. In some preparations, the activation was done at 0°C, in others at 20°C; in either case, the reaction was continued for 10 min while maintaining pH 10.5 - 11.5 with additions of 2 N NaOH. The slurry was poured into a fritted glass filter and washed with about 200 ml 0.1 M sodium carbonate pH 9.0, 0°C. The cake was resuspended in an equal volume of the same buffer. A solution containing 20 mg avidin was added and incubated with the activated Sepharose for 12-24 hr at 4°C. The activated Sepharose was quenched with additional incubation with 0.05 M 2-aminoethanol for 1 hr at 20°C. The avidin-Sepharose was poured into a chromatographic column and successively eluted with 2 vol of HSB, 2 vol of HSUB, 3 vol of 6M GuHCl, pH 2.5, and 5 vol of HSB containing 1 mM EDTA, in which it was stored until use. The effective capacity of the avidin-Sepharose was determined by measuring the amount of ^{14}C-biotin which could be bound to 1 ml of avidin-Sepharose in HSB and eluted with 6M GuHCl. The procedure just described was found to give 5-15 nmole biotin binding sites per ml of packed bed. An alternative synthesis of avidin-Sepharose has been described recently (23).

To facilitate recovery of tRNA-biotin from avidin-Sepharose, the strongest binding sites on the adsorbent were presaturated with free biotin as follows. Columns of avidin-Sepharose were washed with HSB containing a several fold excess of biotin. They were then washed successively as described above with HSB, HSUB and GuHCl, pH 2.5, to liberate biotin from the weaker binding sites. The columns were regenerated in HSB.

Purification of tRNA-biotin on avidin-Sepharose: tRNA-biotin was purified from residual tRNA and tRNA-amine by selective retention on and elution from a 5 ml column of avidin-Sepharose. The sample was loaded in HSB, and the column was washed with 5 vol of the loading buffer. tRNA without biotin does not bind at this ionic strength. The column was then washed with HSUB to eliminate any tRNA retained by nonspecific hydrophobic interactions. This treatment does not disrupt any but the weakest avidin-biotin interactions. The tRNA-biotin was eluted with 6M GuHCl, pH 2.5, identified by scintillation counting and dialyzed against HSB. The avidin-Sepharose columns were regenerated by washing with 5 vol HSB and could be reused at least several times.

DEAE-cellulose chromatography of tRNA-biotin: tRNA-biotin was chromatographed on DEAE-cellulose columns in 7M urea, 10 mM Tris, pH 8.0, and eluted

with a 0.25 M to 0.5 M NaCl gradient according to the method of Penswick and Holley (24). See legend to Fig. 3 for additional experimental details.

Isolation of Ferritin: Ferritin was purified from horse spleen according to a procedure modified from Granick (25). Three or four horse spleens weighing ca. one kg each were minced in a meat grinder and ferritin was extracted from the residue in 5 ℓ of 80°C water for 10 min. After cooling to 5-10°C in an ice-salt bath, the mixture was filtered through cheesecloth. The filtrate was centrifuged 15 min at 1500 x g. The ferritin was then precipitated by adding solid ammonium sulfate to 35% (w/v). After an overnight incubation at 4°C, the precipitate was collected and redissolved in 2% ammonium sulfate. Insoluble material was removed by centrifugation and the supernatant was made 4% $CdSO_4$, by adding ½ vol of 20% Cd SO_4, pH 5.8. Ferritin crystals were collected after 3 hr at 4°C, dissolved in 2% ammonium sulfate and the crystallization procedure repeated 4 or 5 times. Ferritin was precipitated twice with 50% ammonium sulphate, dialyzed extensively versus 50 mM NaP, pH 7.0, and sterilized by passage through Millipore HAWP filters (0.45 µM). Approximately 1 g of ferritin was obtained, which was stored either as an aqueous solution at 2°C or in 50% glycerol, 25 mM NaP, pH 7.0, at -20°C.

Synthesis and purification of ferritin-avidin conjugates: Ferritin (20 mg/ml) in 0.3 M potassium borate, pH 9.3, was bromoacetylated by gentle mixing with about 0.06 vol of a 10 mg/ml solution of the NHS ester of bromoacetic acid in DMSO. After reaction for 1 hr at room temperature, the sample was dialyzed against 1XSPE. The extent of modification was determined by reaction with ^{14}C-cysteine and measurement of the nondialyzable radioactivity.

The addition of sulfhydryl groups to avidin was accomplished with the same chemistry as outlined above. To a solution of avidin (2.0 - 2.4 mg/ml) in 0.3 M potassium borate, pH 9.3, NHS-dithiodiacetic acid dissolved in DMF was added to give a final ester concentration of 1-3 mg/ml. After reaction for several hr at room temperature, sulfhydryl groups were liberated by treatment for 20 min at 37°C with DTT at a concentration of 12-18 mg/ml. Excess ester and DTT were removed from the reaction mixture by dialysis against 1XSPE under argon. The number of SH groups/avidin was assayed by determining the nondialyzable binding of ^{14}C-N-ethylmaleimide.

Ferritin $\left(\overset{O}{\overset{\|}{C}}CH_2Br\right)_n$ was mixed with avidin $\left(\overset{O}{\overset{\|}{C}}CH_2SH\right)_m$ in 0.3 M potassium borate buffer, pH 9.3, under argon. The ferritin concentration was 8.2 - 10.4 µM and the avidin concentration was 21-26 µM. After 2 hr at room temperature, the coupling reaction was quenched by adding 2-aminoethanol (16 M), pH 9.0, to a final concentration of 0.38 M. The mixture was then

layered on 5-50% sucrose gradients containing HSB + 1mM EDTA built on a 60% sucrose cushion. Ferritin-avidin conjugates and free ferritin were separated from free avidin by two cycles of velocity sedimentation at 36,000 rpm for 7 hr in SW 50.1 rotor at -2°C (or at 40,000 rpm for 4 hr at 0°C).

Hybridization of tRNA-biotin to DNA - the ϕ80 psu_3^- system: Phage stocks were prepared as described previously (7). 1.0×10^{10} phage particles of ϕ80 wild type and of ϕ80 psu_3^- (with a $tRNA^{tyr}$ gene) (each sufficient to contribute 0.5 µg DNA) were diluted into 20 µl of 0.2 M EDTA, pH 8.5, and incubated for 10 min at 20°C. 40 µl H_2O and 20 µl of 1M NaOH were added and incubation was continued for 10 min. The solution was neutralized with 30 µl of 2.5 M Tris HCl, pH 3.5. E. coli tRNA-biotin (3-50 µg) was added and the solution was dialyzed versus 40% formamide, 0.3 M NaCl, 0.1 M Tris, 0.001 M EDTA, pH 8.0, for 30-60 min at 40°C. After hybridization, excess tRNA-biotin was removed from the mixture by passage over a 3 ml Sepharose 2B column equilibrated with 1XSPE. The excluded volume was collected and concentrated under vacuum 5- to 7-fold to 50-75 µl.

Labeling DNA:DNA:tRNA-biotin hybrids with ferritin-avidin: Nearly equal volumes of ferritin-avidin and the hybrids were mixed to give a ferritin concentration of 0.1-1 mg/ml (about 10^{-7} - 10^{-6}M), the equivalent of a 1000-10,000-fold excess over the hybridized tRNA-biotin. To allow conjugation, samples were incubated for at least 16 hr at 20°C. Excess ferritin-avidin was removed by centrifuging the mixture through a 5.4 ml gradient of sodium iothalamate (ρ = 1.2-1.4), buffered with 0.1 M Tris, 10 mM EDTA, pH 8.0, for at least 8 hr at 35,000 rpm at 15°C in an SW 50.1 rotor. Since the density of DNA in sodium iothalamate is 1.14 (26), while that of ferritin is estimated to be 1.6 - 1.8 (15), ferritin-labeled hybrids can be separated from excess ferritin-avidin. The DNA-containing fractions (0.2 - 0.6 ml from the top of the gradient) were collected manually from the top, dialyzed against 0.8 M NaCl, 0.1 M Tris, 0.01 M EDTA, pH 8.5, and then against 0.2 M Tris, 0.02 M EDTA, pH 8.5. In some cases the samples were concentrated 3- 4-fold in a vacuum dessicator and redialyzed against 0.2 M Tris, 0.02 M EDTA, pH 8.5, in preparation for electron microscopy.

Electron microscopy: The electron microscopic procedures used here are described in more detail in Davis et al. (1). The spreading solution contained 50% formamide, 0.1 M Tris, 0.01 M EDTA, pH 8.5, and 50 µg/ml cytochrome-c. Depending on the fraction taken from the iothalamate gradient and depending on the experiment, the final DNA concentration ranged from 0.01 - 0.25 µg/ml. The hypophase consisted of 15% formamide, 0.01 M Tris, 0.001 M

EDTA, pH 8.5. The DNA was picked up on parlodion-coated copper grids, stained with 10^{-4} M uranyl acetate and shadowed with 3 - 3.5 cm of platinum palladium (80:20) wire (0.008 gauge) at an angle of 1:9 radians.

Heteroduplex molecules were examined to determine whether they had ferritin labels at the appropriate location on the ϕ80 psu$^-_3$ strand based on previous mapping (7). Labeled molecules were photographed on 35 mm film at a magnification of 4620 and traced with a Hewlett-Packard digitizer to confirm the location. The percentage of labeling was calculated as the number of correctly labeled molecules divided by one-half the number of heteroduplexes observed (since in half the heteroduplexes the ϕ80 psu$^-_3$ strand is not the complement of $tRNA^{tyr}$).

RESULTS

Synthesis of tRNA-biotin: The synthesis of E. coli tRNA-biotin was carried out as described in Experimental Procedures. The oxidation reaction was essentially complete as assayed by nondialyzable binding of ^{14}C-isonicotinic hydrazide. Addition of diaminopentane to form a Schiff base was not quantitative and the yield was highly variable among experiments. In previous studies in which oxidized tRNA was treated with cystamine and the product was reduced with DTT and assayed for SH groups with ^{14}C-N-ethylmaleimide, the yields of tRNA-amine varied from 20-80% (7 and our unpublished observations). Similarly, in these experiments, the yield of tRNA-biotin varied from 20-80% as determined either by using ^{14}C-biotin or by binding the reaction mixtures to avidin-Sepharose columns. Since the acylation of primary amines with NHS esters is known to be very efficient, we believe that the variability in yields of tRNA-biotin results from incomplete formation of the Schiff base and/or of its reduction by $NaBH_4$. We show below that the procedure causes little if any degradation of the tRNA.

tRNA-biotin conjugates were purified from unmodified tRNA on avidin-Sepharose columns prewashed to remove uncrosslinked avidin subunits and preloaded with biotin to mask the strong binding sites as described in Experimental Procedures. The recovery of tRNA from these columns is usually about 95%. An example of the elution profile is illustrated in Fig. 2. In the top panel, the first passage of tRNA-biotin is shown. In this particular preparation, 50% of the A_{260} bound to the column in 1M NaCl and was eluted in 6M GuHCl, pH 2.5. The bottom panel of Fig. 2 shows that greater than 95% of this material bound on repassage, indicating that the tRNA was not degraded as a result of exposure to the strongly denaturing elution buffer. Further, the excellent rebinding of the tRNA-biotin conjugates suggests that

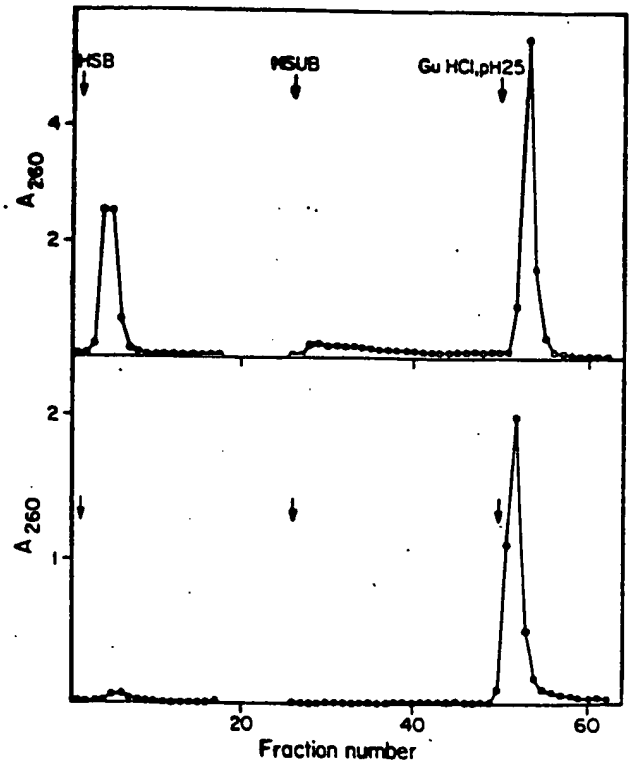


Fig. 2. Affinity chromatography of tRNA-biotin on Avidin-Sepharose. Top: 900 µg (32 nmoles) of tRNA in HSB was loaded on a 5 ml column of avidin-Sepharose. The total biotin binding capacity of the column was 45 nmoles. Twenty-five ml each of HSB, HSUB and 6 M GuHCl, pH 2.5, were passed through the column. One ml fractions were collected. Bottom: A portion of the material which bound to avidin-Sepharose was rechromatographed on the same column using exactly the same procedure.

it is unlikely that avidin subunits were released during the first pass and became attached to tRNA-biotin.

In one experiment, tRNA-amine was acylated with NHS-^{14}C-biotin (Sp. act. = 1.4 x 10^7 cpm/µmole). After purification on avidin-Sepharose, the specific activity of the tRNA-biotin conjugates was 6.2 x 10^2 cpm/µg, indicating that the biotin:tRNA ratio was 1.1I:1. Assuming that acylation occurred only at the 3' termini and the tRNA was not degraded, this ratio indicates that the tRNA-biotin preparation is quite pure.

In order to check that the tRNA-biotin was not degraded during the derivatization and purification procedures, it was chromatographed on DEAE-cellulose

in 7M urea, 10 mM Tris, pH 8.0 and eluted with a linear NaCl gradient from 0.25M-0.5M following the procedure of Penswick and Holley (24). According to their results and ours, intact tRNA elutes at ca. 0.38 M NaCl while half size molecules elute at lower ionic strengths (0.31M-0.35M). As shown in Fig. 3, 98% of the tRNA-biotin elutes in a single peak at about 0.38 M indicating that most of the molecules are still full size, or nearly so, and therefore long enough to form stable hybrids. These results also indicate that avidin subunits do not leak from the column and bind to tRNA-biotin. If avidin (pI = 10.5) were bound to tRNA-biotin, the elution profile of such complexes which are stable in 7M urea (27) would be different from tRNA alone. Other RNA-biotin samples were analyzed by electrophoresis on 6M urea-15% polyacrylamide gels. No degradation could be detected when the electrophoresis profiles of RNA-biotin and unmodified RNA were compared.

Ferritin-avidin Coupling. Table I shows that, in four separate preparations of bromoacetylated ferritin, between 10 and 21 moles of active bromide were added per mole of ferritin as assayed by the non-dialyzable binding of ^{14}C-cysteine (see footnote a of Table I for details of assay). Attachment of SH groups to avidin was carried out as described in Experimental

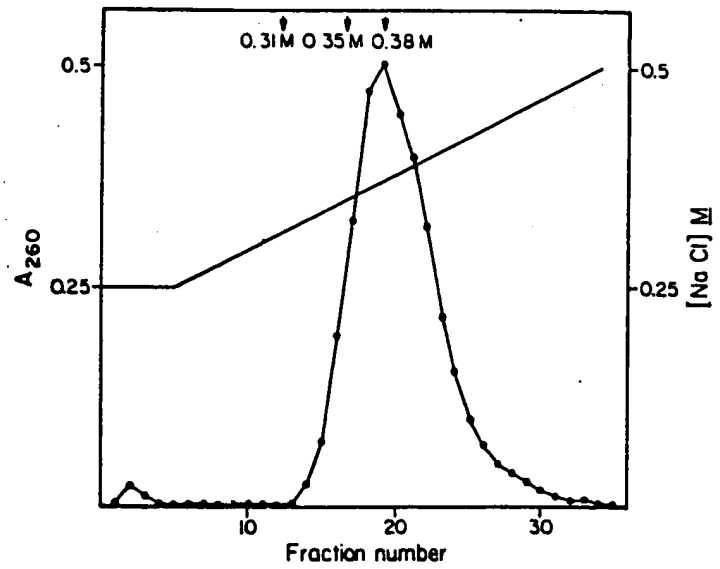


Fig. 3. Chromatography of tRNA-biotin on DEAE-cellulose in 7M urea. 360 µg of tRNA-biotin was dissolved in 7M urea, 10 mM Tris, pH 8.0, 0.25M NaCl and loaded on a 2.3 ml column of DEAE-cellulose. The column was developed with a 50 ml gradient from 0.25-0.5 M NaCl. 0.7 ml fractions were collected.

Table I

Synthesis of Bromoacetylated Ferritin and Avidin-SH

Experiment Number	Input Molar Ratio NHS ester / ferritin	Input Molar Ratio NHS ester / avidin	Reaction Time (hours)	± DTT	Molar ratios (a) ^{14}C cysteine bound / ferritin	Molar ratios (b) ^{14}C NEM / avidin	Molar ratios (c) ^{14}C biotin / avidin
1	100	-	1	-	16.3	-	-
2	120	-	2.5	-	20.8	-	-
2a	10	-		-	0.3	-	-
3	105	-		-	10.1	-	-
4	95	-	2	-	12.6	-	-
1	-	100	1	+	-	15.0	3.2
2	-	290	1	+	-	10.8	1.9
3	-	100	2	+	-	5.5	2.0
4a	-	100	2	+	-	5.8	4.0
4b	-	0	0	+	-	1.4	3.6
4c	-	0	0	-	-	-	3.7

(a) Bromoacetylated ferritin was assayed by incubatint 10 to 20 piomoles of either modified or unmodified ferritin with 25 nmoles of ^{14}C cysteine (Sp. Act. = 15 C/mole) in 50 µl of 0.3 M potassium borate, pH 9.3 under argon for 2 hrs at 25°C. Excess cysteine was removed by dialysis vs. 1XSPE, 1mM DTT.

(b) Avidin-SH was assayed by incubating 0.7 to 0.9 nmoles of either modified or unmodified avidin with 50 nmoles of ^{14}C N-ethylmaleimide (NEM)(Sp. act. = 2.5 C/mole) in 200 µl of 1XSPE, pH 7.5, for 2 hrs at 25°C. Excess NEM was removed by dialysis versus 1XSPE.

(c) Biotin binding was determined by incubating 0.7 to 0.9 nmoles of either modified or unmodified avidin with 30 nmoles of ^{14}C biotin (Sp. act. = 45 C/mole) in 100 µl of 1XSPE for 1 hr at 25°C. Excess biotin was removed by dialysis versus 1XSPE.

Procedures. After liberation of the sulfhydryl groups with DTT, the preparations were dialyzed extensively in oxygen-free buffer and assayed with ^{14}C-N-ethylmaleimide (see footnote b of Table I for details of assay). Although the number of sulfhydryl groups added was somewhat variable among different experiments, all preparations were still capable of binding biotin. In some preparations, no loss of biotin binding was observed (see footnote c, Table I for assay). All of these preparations were able to couple to bromoacetylated ferritin with roughly similar efficiencies.

Modified ferritin and avidin were coupled and purified as described in Experimental Procedures. After the first sucrose gradient sedimentation, 70% of the total biotin binding activity was recovered; the remainder apparently pelleted in insoluble ferritin-avidin aggregates. 65% of the recovered biotin binding activity was associated with the ferritin band in the lower third of the gradient while the remaining 35% was found at the top of the gradient with uncoupled avidin-SH. The fractions containing ferritin were pooled, dialyzed and run on a second identical sucrose gradient and the biotin binding activity of various portions of the gradient determined. 99.9% of the biotin binding activity sedimented with the ferritin band. When

several fractions within the ferritin band were assayed with ^{14}C-biotin, the number of moles of biotin bound per mole of ferritin varied from 2.5 to 4.8, values which correspond to the slower and faster sedimenting ferritin-avidin conjugates, respectively. Electron microscopic examination of these fractions showed that the faster sedimenting material contained more aggregates while the slower sedimenting material was almost entirely ferritin monomers. The monomer fractions were used for the gene labeling experiments. We estimate, both from the recovery of biotin binding activity in these gradients and the number of moles of biotin bound per mole of ferritin, that an average of one to two moles of avidin have been coupled to each mole of ferritin.

The following experiment was done to test whether these ferritin-avidin conjugates could bind tRNA-biotin. Equimolar amounts of tRNA-^{14}C-biotin (0.65 nmoles, 6900 cpm) and ferritin-avidin (0.65 nmoles ferritin, 2.6 nmoles biotin binding sites) were incubated in 1 x SPE for 24 hours at room temperature. In an identical control reaction, 0.8 nmoles of ^{3}H tRNA (3.47 x 10^{4} cpm) were incubated with 0.65 nmoles of ferritin-avidin. The reaction mixtures were sedimented 4 hrs at 40,000 rpm at 0°C in the SW 50.1 rotor through a 5-20% sucrose gradient built on a 0.5 ml 60% sucrose cushion containing 2XSPE. As shown in Fig. 4, 100% of the counts in the control reaction (•—•), were recovered and sedimented near the top of the tube while in the tRNA-biotin:avidin-ferritin reaction (x—x), 91.8% of the counts were found associated with the ferritin band in the bottom two fractions of the gradient. Since no absorbance due to tRNA (Δ—Δ) was detected at the top of this gradient, we conclude that the ^{14}C counts at the bottom of the gradient represent the binding of at least 90% of the tRNA-biotin in the reaction. Other experiments confirm that our preparations of ferritin-avidin contain little if any ribonuclease activity, since ^{3}H-tRNA incubated with ferritin-avidin as in the control experiment described above remains full length as assayed in denaturing polyacrylamide gels (data not shown).

Gene Mapping Studies. In order to test the efficiency of labeling DNA:tRNA-biotin hybrids with ferritin-avidin, we have used the heteroduplex formed between the bacteriophage DNAs of ϕ80 wild type and ϕ80 psu_3^-. ϕ80 psu_3^- contains a 3.2 kb sequence of E. coli DNA carrying one gene for tyrosine tRNA. The position of this gene in the ϕ80 wild type/ϕ80 psu_3^- heteroduplex was mapped in previous studies (7). The hybridization conditions and methods for purifying the hybrids are described in detail in Experimental Procedures. Fig. 5a is an electron micrograph of a heteroduplex labeled with ferritin

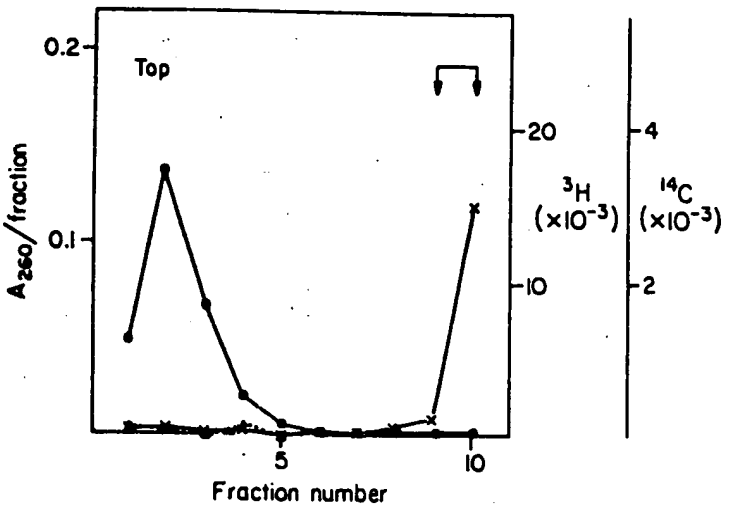
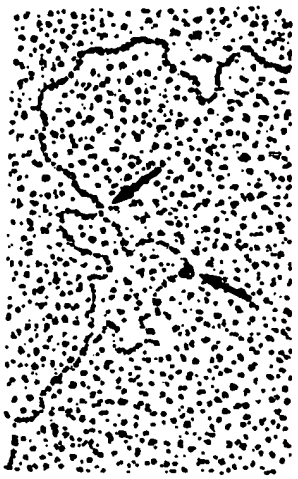


Fig. 4. 5-20% Sucrose gradient sedimentation of reaction mixtures containing either ^{3}H-tRNA + ferritin-avidin (•-•) or tRNA-^{14}C-biotin + ferritin-avidin (x-x). The A_{260} due to tRNA-biotin is also indicated (Δ-Δ). Ferritin sedimented to the bottom of the gradient as indicated by the arrow. Note that the concentration of tRNA at the bottom of the gradient cannot be measured by A_{260} because of the large absorbance by ferritin at this wave length.

in the proper position. A histogram of the distribution of labels is presented in Fig. 5b. In this experiment, 106 heteroduplexes were scored for labels. Thirty three heteroduplexes contained ferritin bound to the tRNA gene which is located 1200 nucleotides to the right of the substitution junction at the λ att site. One ferritin was judged to be attached non-specifically. Four ferritins were bound at a position 200 nucleotides to the right of the main peak and, therefore, were not attached to the $tRNA^{tyr}$ gene. This non-random distribution may or may not reflect some weak interaction between the tRNA and DNA at this point.

Several labeling experiments were performed by three different investigators. The combined data are listed in Table II. In these experiments, the hybridizations were carried out over a 25-fold range in rot (rot = (RNA concentration in nucleotide/moles/ℓ) X (time in sec.)). O. Uhlenbeck and his co-workers (personal communication) have determined that the $rot_{\frac{1}{2}}$ for a pure tRNA under the same conditions is 3×10^{-4}. The lowest rot used based on total tRNA concentration was 0.32, or a rot of 0.016 for $tRNA^{tyr}$, if this species constitutes about 1/20th of the total, and thus 50 times greater than the required $rot_{\frac{1}{2}}$. It is clear that there is no correlation between the rot achieved during



(a)

(b)

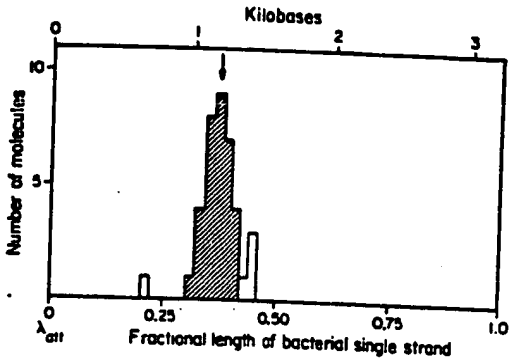


Fig. 5. (a) Electron micrograph of ϕ80 wild type/ϕ80 psu_3^- heteroduplex. The arrows indicate the positions of the ferritin label and the ϕatt site. (b) Histogram of distribution of ferritin labels on the 3.2 kb bacterial segment of ϕ80 psu_3^-, measured to the right from the att junction near the center of the molecule (7). The att site and the fork at the other end of the substitution loop are 23.8 and 19.3 kb from the left and right ends of the heteroduplex, respectively (5); therefore, they are readily distinguished.

Table II

Efficiency of Gene Labeling

Experiment Number	(a) $tRNA^{tyr}$ rot	Ferritin-avidin µg/ml	Ferritin-avidin per gene	Labeling time	Number Hetero-duplexes	Number genes labeled	(b) % genes labeled
1	1.6×10^{-2}	259	9400	17	42	9	22
2	3.4×10^{-2}	1000	5300	18	25	6	48
3	10×10^{-2}	73	4900	16	106	33	62
4a	42×10^{-2}	16	140	8	25	0	0
4b	42×10^{-2}	9.0	790	8	25	0	0
4c	42×10^{-2}	161	14000	70	25	5	40
4d	42×10^{-2}	211	18500	70	27	8	59

(a) The concentration $tRNA^{tyr}$ is assumed to be equal to 1/20 of the mass of tRNA.

(b) $$\% \text{ genes labeled} = \frac{\text{number of labels}}{(\frac{1}{2})(\text{number of heteroduplexes})}$$

the hybridization and the labeling efficiency. The concentration of ferritin-avidin and the ferritin-avidin:gene ratio were also varied. Only in experiments 4a and 4b (Table II) in which both the ferritin-avidin concentration and ferritin-avidin/gene ratio were low was no labeling observed. In the

other experiments the ferritin concentrations were considerably higher. However, aside from this observation, no clear relationship exists between the gene labeling efficiency and either the concentration or molar excess of ferritin-avidin in the labeling reaction.

Discussion and Experimental Precautions. Several experimental precautions should be observed to maximize the efficiency of this gene labeling procedure. Also some potential side reactions may influence the success of the technique.

Preparations of tRNA may be depleted in certain labile species such as $tRNA^{trp}$ and should be tested for amino acid acceptance by the species to be mapped. Obviously, the existence of isoacceptors reduces the value of this assay unless tRNA samples are fractionated. tRNA preparations may be contaminated with 5S and other small RNAs (and vice versa). Usually these possibilities can be tested by appropriate controls.

tRNA is treated with 2M Tris buffer at pH 8.2 to deacylate any residual amino acids (19); thereafter amine buffers must be absent until the chemical linkage of biotin to the RNA is complete. Excess small molecule reagents such as Tris HCl, $NaIO_4$, alkane diamines, and NHS-biotin are preferably removed by dialysis or by gel filtration. (Ethanol precipitation of the tRNA does not eliminate all traces of these reagents.) To reduce the possibility of side reactions, solvents and pH are not altered until the reagents are removed. Oxidized tRNA is particularly sensitive to amine-catalyzed beta-elimination of the 3'-terminal nucleotide (28).

Reactions and incubations should be performed in the dark. For instance, periodate-oxidized tRNA dialdehyde is sensitive to photooxidation to the carboxylic acid. The alkane diamines appear to be light-sensitive and should be stored in the dark. The tryptophan residues at the active site of avidin are sensitive to photooxidation in the presence of Fe^{+3} (12), which is a perpetual contaminant once avidin and ferritin have been mixed.

The formation of the Schiff base between oxidized tRNA and the diamine must be driven by as large an excess of amine as is practicable. Diamines shorter than C5 do not provide a linker arm long enough to allow biotin to extend sufficiently far from the macromolecule carrier surface to penetrate the avidin binding sites (29). Diamines longer than about C7 are not sufficiently soluble in water to allow the Schiff base formation to be driven by the necessary concentration excess. Borohydride reduction of the Schiff base presents the opportunity for introduction of a 3'-terminal tritium label from ^3H-$NaBH_4$, allowing the RNA to be traced subsequently during

chromatography and the stability of tRNA-biotin:avidin-ferritin conjugates to be measured.

Several side reactions may cleave certain tRNA species during the modification and purification steps. In particular, the $NaBH_4$ reduction step modifies 7-methylguanosine and dihydrouridine residues so that adjacent phosphodiester linkages are sensitive to cleavage at low pH (30, 31). E. coli $tRNA^{tyr}$ contains a 7-methylguanosine at position 17 from the 5' end. Cleavage at this point would leave a fragment of tRNA extending 68 nucleotides from the biotin-labeled 3' end. This fragment is of sufficient length to chromatograph and hybridize like intact tRNA in the tests described above. Thus, we do not have a critical test to determine whether the present procedure causes significant cleavage at 7-methylguanosine residues. Periodate oxidation probably converts thiouridine to uridine which is not harmful (32). Whether or not the reduction by $NaBH_4$ of dihydrouridine (32) is deleterious for the present mapping procedure is not known.

Avidin, and particularly avidin-biotin complexes are extraordinarily stable and can withstand heat, pH extremes (2 - 12), urea and 50% formamide (12, 27, 33). The avidin subunits can be separated and the complexes can be denatured in the presence of 6M GuHCl, pH 2.5 or lower (36, 22). Subunit renaturation and reassociation and avidin-biotin binding are freely reversible upon removal of the GuHCl as long as thiol reagents (e.g., DTT) are not present during the denaturation of the avidin (35).

Avidin-biotin complexes are far more stable to subunit dissociation and to denaturation than is free avidin (35). Thus, before exposure to biotin freshly made avidin-Sepharose must be washed with GuHCl, pH 2.5, to remove avidin subunits which were not covalently cross-linked to the matrix. Avidin subunits retain a substantial affinity for biotin (37, 22). Avidin and avidin subunits have a spectrum of biotin affinity constants and it is impractical to reverse the tightest binding (22). Therefore, Sepharose-avidin subunit columns should be exposed first to free biotin to saturate all sites and then eluted with GuHCl, pH 2.5, to free all but the strongest biotin binding sites. These pretreatments will allow good recoveries of tRNA-biotin from the columns and will eliminate any uncrosslinked avidin subunits that might otherwise contaminate the tRNA-biotin upon subsequent elution. Avidin-Sepharose columns are run in the presence of 1 M NaCl at all times to minimize electrostatic interactions between nucleic acids and the very basic avidin.

Ferritin is a somewhat unstable protein and should be freshly recrystal-

lized before use. It does not withstand freezing or exposure to high salt (e.g., 6 M CsCl). We have noticed that it tends to denature upon prolonged exposure to formamide. Extensive treatment with EDTA may cause substantial release of Fe^{+3}, which may adversely affect avidin (12), nucleic acids and R-SH + R-Br reactions. The free iron concentration should be minimized by dialysis of ferritin solutions shortly before reaction or conjugation steps. To insure the absence of apoferritin and unbound avidin, ferritin-avidin preparations should be sedimented through sucrose gradients immediately prior to use. To prevent pelleting the ferritin-avidin, which is then difficult to resuspend, a cushion of 60% sucrose is used at the bottom of the gradient. The gradient is run at approximately 0 to -2°C to increase its viscosity.

Since labeling of DNA:RNA-biotin should be done at high ferritin-avidin concentrations, it is necessary that ferritin-avidin preparations be absolutely free of unbound avidin. The long incubation opens the possibility for nuclease or protease action and compels care in sterile handling throughout preparation of the sample.

Tolerable background concentrations of ferritin can be achieved if the complexes are prepared for electron microscopy at no more than 1.0 µg/ml free ferritin-avidin in the hyperphase spreading solutions. Separation of excess ferritin-avidin from hybrids on sodium iothalamate gradients achieves this level of purity.

Ferritin can be confused easily with coarse platinum shadow. Best definition of the label is achieved if a shutter is imposed between the metal filament and the specimen grids during the initial phase of melting and if subsequent evaporation is done just above the melting temperature of the Pt or Pt-Pd. Very light shadowing is required.

FURTHER DISCUSSION

Unlike studies of the distribution of targets such as proteins in surfaces, identification of single features such as genes or proteins on linear chromosomes requires a high labeling efficiency as well as low backgrounds.

The overall efficiency of labeling achieved here is about 40-50%. The percentage of the genes labeled depends upon the $tRNA^{tyr}$:DNA hybridization and the ferritin-avidin binding efficiencies. It seems plausible that attachment of biotin to tRNA does not significantly alter its rate of hybridization or the stability of the hybrid; therefore, saturation of the gene should be possible at a sufficiently high rot. It is probable that the overall efficiency of this technique is limited by our ability to detect labeled hybrids and by those factors which influence the association of ferritin-

avidin conjugates with biotin-containing hybrids. Such factors include: (1) the stability of ferritin (if iron is lost from ferritin during the course of the experiment, then apoferritin-avidin complexes will be produced which are not visible in the electron microscope); (2) the stability of ferritin-avidin conjugates (traces of free avidin or avidin subunits may react faster with tRNA-biotin hybrids than ferritin-avidin does); (3) the purity of the tRNA-biotin preparation; (4) the stability of the tRNA-biotin linkage; and (5) steric factors which affect the association constants and/or the rates of reaction of ferritin-avidin with biotin-containing hybrids.

We believe that the first three of these factors are not the main cause of the limited labeling efficiency. First, we have used only ferritin-avidin conjugates which have been selected for high density by two cycles of sucrose gradient sedimentation. Second, after the second cycle, we detect less than 0.1% of the total biotin binding activity not associated with ferritin band. Finally, we have shown that tRNA-biotin derivatives are at least 95% pure by repassage through avidin-Sepharose. These derivatives contain an equimolar ratio of biotin to tRNA. The RNA remains full size or nearly full size during modification.

Earlier experiments have shown that the tRNA-amine linkage is stable to our hybridization conditions (7). This result is confirmed with tRNA-biotin by the observation that tRNA-biotin first incubated under hybridization conditions binds ferritin-avidin as extensively as unincubated control samples. Thus, we expect that the tRNA-biotin linkage is stable during our experiments.

Some steric problem which interferes with the avidin reaction might contribute to suboptimal labeling efficiencies. We have found that very high concentrations of ferritin-avidin are required to drive the labeling reaction although much lower concentrations are sufficient to bind unhybridized tRNA-biotin. This observation may be explained by considering the following ideas. First, tRNA-biotin may have a less favorable association with ferritin-avidin than does free biotin. If the linker bridge between biotin and the macromolecule to which it is coupled is not at least 7 or 8 bonds, the equilibrium constant will increase markedly (36, 29). Second, while the chemistry used to modify avidin for subsequent reaction with ferritin does not significantly reduce the number of biotin binding sites, the effect on the equilibrium constant is not known. Finally the concentration of avidin:biotin complex in the spreading solution is very low, about 10^{-11} M. Therefore, the conjugate must be exceptionally stable. With equilibrium binding constants much greater than 10^{-13} M, substantial dissociation would occur in the

time necessary for removal of excess ferritin-avidin or even for spreading the hyperphase during electron microscopic grid preparation. The equilibrium constant for free avidin and biotin at pH 7 in high salt (0.1 M NaCl) is about 10^{-15} - 10^{-13} M (13, 22). If the tRNA-biotin:avidin-ferritin association is weaker by several orders of magnitude, perhaps only a small fraction of ferritin-avidin conjugates are capable of forming stable associations under our labeling and spreading conditions.

With this caveat, the avidin-biotin affinity pair satisfies all requirements for speed and stability of association, for availability of materials, for ease of attachment to a wide variety of macromolecules and solid supports, and for convenient assay. All necessary reactions that might affect the integrity of biological materials can be done between pH 5 and 9 and at temperatures no higher than 37°C in aqueous or compatible mixed solvents. The reactions are versatile and strategies can be devised and executed in which either avidin or biotin can be coupled to any of the components which must be conjugated. For example, in addition to gene mapping studies, the avidin:biotin linkage has recently been used to enrich the 18s and 28s ribosomal genes (38, 39) and the 5s ribosomal genes from whole Drosophila DNA (9).

In contrast to a method previously described (7), in which ferritin was conjugated directly to tRNA before its hybridization to DNA, the scheme reported here allows the label to be added to established DNA:tRNA-biotin hybrids. In principle, this should allow more efficient nucleic acid hybridization and higher labeling efficiencies. Furthermore, the ferritin label is added after the hybridization in a nondenaturing solvent which preserves ferritin structure. In practice, the maximum efficiencies of both methods are about 60%, but the ease of handling, the speed and the reproducibility in the hands of many workers are higher with the avidin-biotin mediated linkage than with the chemical linkage.

It is important to emphasize that the labeling efficiencies achieved here allow analysis only of defined segments of DNA. Specifically, this means that a fixed point or marker is needed from which to map the labeled genes. Such a reference point could be a restriction endonuclease cleavage site, a substitution or deletion loop in a heteroduplex, a secondary structure feature in the DNA or a long duplex region formed by hybridizing specific RNA or DNA probes (8, 40). When such systems are studied with this technique, it is possible in a single experiment to obtain a gene map with a resolution of several hundred nucleotides.

ACKNOWLEDGMENTS

We gratefully acknowledge our colleagues Drs. Louise T. Chow, Maria Pellegrini, and Chin Hua Wu for helpful discussions and experimental contributions to the development of these techniques. TRB was supported by a Helen Hay Whitney Fellowship, LMA by a Damon Runyan Fellowship, and NDH by an NSF Fellowship. General research support was received from an NIH grant to ND.

REFERENCES

1. Davis, R.W., Simon, M., and Davidson, N. (1971) Methods in Enzymology 21D, 413-428.
2. Ohtsubo, E., Lee, H-J., Deonier, R.C., and Davidson, N. (1974) J. Mol. Biol. 89, 599-618.
3. Forsheit, A.B., Davidson, N., and Brown, D.D. (1974) J. Mol. Biol. 90, 301-315.
4. Thomas, M., White, R., and Davis, R.W. (1976) Proc. Nat. Acad. Sci. USA 73, 2294-2298.
5. Wu, M. and Davidson, N. (1975) Proc. Nat. Acad. Sci. USA 72, 4506-4510.
6. Reed, S.I. and Alwine, J.C. (1977) Cell 11, 523-531.
7. Wu, M. and Davidson, N. (1973) J. Mol. Biol. 78, 1-21.
8. Angerer, L., Davidson, N. Murphy, W., Lynch, D. and Attardi, G. (1977) Cell 9, 81-90.
9. Sodja, A. and Davidson, N. (1978) Nuc. Acid. Res. , following paper.
10. Manning, J.E., Hershey, N.D., Broker, T.R., Pellegrini, M. and Davidson, N. (1975) Chromosoma (Berl.) 53, 107-117.
11. Green, N.M. and Toms, E.J. (1970) Biochem. J. 118, 67-70.
12. Fraenkel-Conrat, H., Snell, N.S., and Ducay, E.D. (1952a) Arch. Biochem. Biophys. 80, 80-96.
13. Green, N.M. (1963a) Biochem. J. 89, 585-591.
14. Heitzmann, H. and Richards, F.M. (1974) Proc. Nat. Acad. Sci. USA 71, 3537-3541.
15. Fischbach, F.A. and Anderegg, R.W. (1965) J. Mol. Biol. 14, 458-473.
16. Becker, J.M., Wilchek, M. and Katchalski, E. (1971) Proc. Nat. Acad. Sci. USA 68, 2604-2607.
17. Jasiewicz, M.L., Schoenberg, D.R., and Mueller, G.C. (1976) Experimental Cell Research 100, 213-217.
18. Pellegrini, M., Oen, H., and Cantor, C.R. (1972) Proc. Nat. Acad. Sci. USA 69, 837-841.
19. Sarin, P.S., and Zamecnik, P.C. (1964) Biochim. Biophys. Acta 91, 653-655.
20. Axen, R., Porath, J., Ernback, S. (1967) Nature 214, 1302-1304.
21. Bodanszky, A. and Bodanszky, M. (1970) Experientia 26, 327.
22. Green, N.M. and Toms, E.J. (1973) Biochem. J. 133, 687-698.
23. Hofmann, K., Finn, F.M., Friesen, H-J., Diaconescu, C., and Zahn, H. (1977) Proc. Nat. Acad. Sci. USA 74, 2697-2700.
24. Penswick, J.R. and Holley, R.W. (1965) Proc. Nat. Acad. Sci. USA 53, 543-546.
25. Granick, S. (1946) Chem. Revs. 38, 379-403.
26. Serwer, P. (1975) J. Mol. Biol. 92, 433-448.
27. Green, N.M. (1963b) Biochem. J. 89, 599-609.
28. Fraenkel-Conrat, H. and Steinschneider. A. (1968) Methods in Enzymology 12B, 243-246.
29. Green, N.M. Konieczny, L., Toms, E.J. and Valentine, R.C. (1971) Biochem. J. 125, 781-794.

30. Wintermeyer, W. and Zachan, H.G. (1975) FEBS Letters 58, 306-309.
31. Corutti, P. and Miller, N. (1967) J. Mol. Biol. 26, 55-56.
32. Von der Haar, F., Schlimme, E., and Gauss, D.H. (1971) In Procedures in Nucleic Acid Research, vol. 2, p. 643-664.
33. Gyorgy, P., Rose, C.S. and Tomarelli, R. (1942) J. Biol. Chem. 144, 169-173
34. Fraenkel-Conrat, H., Snell, N.S., and Ducay, E.D. (1952b) Arch. Biochem. Biophys. 80, 97-107.
35. Green, N.M. (1963c) Biochem. J. 89, 609-620.
36. Cuatrecasas, P., and Wilchek, M. (1968). Biochem. Biophys. Res. Comm. 33, 235-239.
37. Green, N.M. and Ross, M.E. (1968) Biochem. J. 110, 59-66.
38. Manning, J., Pellegrini, M., Davidson, N. (1977) Biochem. 16, 1364-1370.
39. Pellegrini, M., Holmes, D.S. and Manning J. (1977), Nuc. Acids Res. 4, 2961-2974.
40. Yen, P.H., Sodja, A., Cohen, M. Jr., Conrad, S.E., Wu, M., Davidson, N. and Ilgen C. (1977) Cell 11, 763-777.